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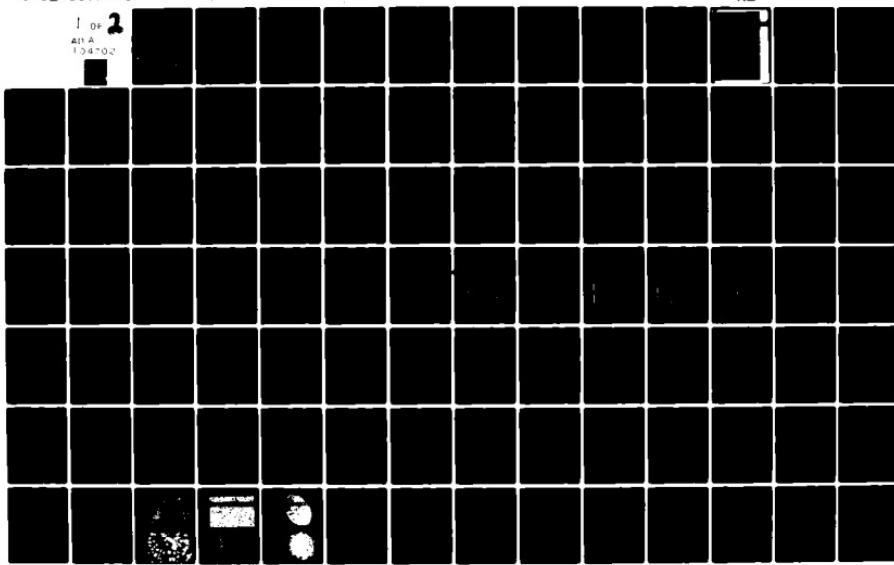
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FINAL TECHNICAL REPORT

OXYGEN TOXICITY AND LUNG COLLAGENOUS PROTEIN

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Principal Investigators: Klaus Brendel, Ph.D. and I. Glenn Sipes, Ph.D.
Departments of Pharmacology and Toxicology

Arizona Health Sciences Center
University of Arizona
Tucson, AZ 85724

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INTRODUCTION

The major objective of this proposal was to investigate the chemical composition and subunit structure of the basement membrane and collagenous components of the normal lung and of lungs exposed to agents thought to promote the formation of singlet oxygen and thereby produce oxidative damage to tissue components. The pathological and functional changes attributable to singlet oxygen production can be induced experimentally by paraquat (1,1-dimethyl-4, 4-dipyridilium dichloride) which is thought to produce toxicity comparable to that produced by hyperoxia.

Connective tissue makes up about 25% of the adult human lung and plays an important role in the mechanical and physiological functions of the lung. The alveolar basement membrane, because it lies in the primary pathway traversed by ventilated gases, is of particular interest and, therefore, the determination of the structure and composition of this component of the extracellular matrix of the lung is of considerable importance. In the last few years, it has become increasingly obvious from the study of extracellular matrix from a variety of tissues that a complex population of proteins make up the extracellular matrix. Several new collagen types have been described as well as high molecular weight non-collagens that appear to be associated with normal basement membranes. In view of the new and somewhat unanticipated information accumulating in this area, the characterization of putative changes in the nature of matrix components in pathologic states must, of necessity, lag behind a more thorough characterization of normal basement membrane and extracellular components.

The lung is also made up of a complicated population of different cell types, each of which may respond differently to toxic substances. The interpretation of toxicological studies of relevance to pulmonary function would be greatly facilitated by the development of an in vitro system allowing the use of defined

pulmonary cell type of major physiological importance such as the type II pneumocyte. Accordingly, this aspect of the project was pursued vigorously.

This report summarizes the work performed during this contract by providing a brief introduction to the various experiments followed by the pertinent data in the form of the resulting publication or manuscript.

EFFECTS OF PARAQUAT

Paraquat is a broad spectrum herbicide that has a very toxic affinity for the lung, although with some indications of toxicity for the kidney as well. Single oral or injected doses result in hemorrhagic pulmonary edema within a few hours followed by an irreversible inflammation and fibrosis detectable morphologically. The effects of paraquat treatment on prolyl hydroxylase activation and collagen synthesis were determined in lung and kidney slices and cultured lung cells taken from rats exposed to paraquat nine days prior to sacrifice. The results are presented in the following manuscript:

Kuttan, R., Lafranconi, M., Sipes, I.G., Meezan, E. and Brendel, K. (1979). Effect of paraquat treatment on prolyl hydroxylase activity and collagen synthesis of rat lung and kidney. Res. Comm. Path. Pharm. 25, 257-268.

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EFFECT OF PARAQUAT TREATMENT ON PROLYL HYDROXYLASE ACTIVITY
AND COLLAGEN SYNTHESIS OF RAT LUNG AND KIDNEY

R. Kuttan, M. Lafranconi, I.G. Sipes, E. Meezan
and K. Brendel

Departments of Pharmacology and Anesthesiology
The University of Arizona Health Sciences Center,
Tucson, Arizona 85724

ABSTRACT

Morphological evidence has shown that the herbicide paraquat produces severe lung fibrosis in experimental animals. In order to investigate the biochemical basis for this fibrosis, prolyl hydroxylase activity, total hydroxyproline content and collagen and protein synthesis were estimated in rats (200 gm) which were pretreated with paraquat (25 mg/Kg; I.P.) nine days before sacrifice. Prolyl hydroxylase activity in the treated rats was significantly elevated in the lungs. However, no such increase was seen in the kidney. There was no significant change in the total hydroxyproline content or collagen formation in the treated animals. When paraquat was added to the culture medium and incubated with lung slices, there was a marked decrease in collagen synthesis along with a decrease in protein synthesis. When paraquat was added to lung cells in culture, it was found that lung explants were more sensitive to paraquat toxicity than cells obtained by trypsinization. In explants which were sensitive to paraquat, there was a marked decrease in both protein synthesis and collagen synthesis.

INTRODUCTION

Morphological evidence suggests that the herbicide paraquat produces severe lung fibrosis in human beings who have ingested this chemical accidentally as well as in experimental animals (Vijeyaratnam and Corrin, 1971; Smith, 1976). Even though the lung has a capacity to concentrate paraquat (Rose et al., 1974), pathological changes have also been seen in kidney and liver (Gibson and Cagen, 1977). The primary effect of paraquat in animals has been shown to be the destruction of type I alveolar epithelial cells (Kimbrough and Gaines, 1970). Damage to granular

pneumocytes (type II cells) and endothelial cells was minimal (Vijeyaramam and Corrin, 1971; Kimbrough and Linder, 1973). This suggests a selective action of paraquat on different cell lines depending upon the rate of absorption and metabolism of paraquat by the cells. However, the effect of paraquat on culture systems has not been studied. Such an approach can yield interesting data on the action of paraquat.

Paraquat induced lung fibrosis has been shown to result in deposition of collagen (Smith et al., 1974). Studies on other systems have shown that prolylhydroxylase can be used as a biochemical marker for collagen synthesis (Cardinale and Udenfriend, 1974). Collagen synthesis can also be estimated by the incorporation of radioactive proline into radioactive hydroxyproline or into collagenase degradable protein. Using these biochemical markers, the effect of paraquat on collagen synthesis in the lung and kidneys of whole animals was measured nine days after administration of an acute dose of paraquat (25 mg/Kg). At this time the lung tissue is in a proliferative phase of repair of the acute injury induced shortly after paraquat administration. A tissue culture system has been employed to study the effect of paraquat on collagen synthesis of isolated lung and kidney slices and isolated explants and cells obtained by trypsinization from neonatal lungs.

MATERIALS AND METHODS

Paraquat dihydrochloride (Imperial Chemicals) was a gift from Dr. L. Smith. ¹⁴C-proline was obtained from New England Nuclear. Tissue culture media were obtained from GIBCO.

Both neonatal and adult rats were used in the experiments. Paraquat was administered to the adult rats (200 gm) by intraperitoneal injection (25 mg/Kg). The rats were sacrificed after nine days by exsanguination.

Lung cells were prepared from neonatal rats by two techniques, an explant method and by trypsinization. To start the cells from explants, thin slices of the neonatal rat lungs were placed on nylon grids in a petri dish containing Dulbecco's modified Eagles medium with 10% fetal calf serum and antibiotics (penicillin 100 units/ml, streptomycin 100 µg/ml and fungizone 0.25 µg/ml). Trypsinization of the lung cells was done using 0.1% trypsin for 1 hr as described by Hance et al. (1976). After trypsinization, the cells were washed in phosphate buffered saline pH 7.4 and were grown with an initial cell density of 0.2 million/ml in the same medium described before.

Collagen synthesis was measured using lung and kidney slices (100 mg) employing a procedure involving treatment with a highly purified form of bacterial collagenase (Peterkofsky and Diegelmann, 1971). This was done as follows: medium was changed to a fresh medium (5 ml) without serum but containing antibiotics. The following mixture was added to the medium; sodium ascorbate (10^{-4} M), ^{14}C -proline (2 μc) and β -amino propionitrile (20 µg/ml) which inhibits cross-linking of the newly formed collagen. The incubation was performed at 27°C for 48 hrs. The tissue was separated from the medium and was homogenized in Tris buffer (0.05 M, pH 7.6) containing ethylene diamine tetracetic acid (10^{-5} M), dithiothreitol (10^{-4} M) and Triton X-100 (0.1%). Part of the homogenate was dialyzed against Tris buffer (0.01 M, pH 7.6) overnight. Incorporation into collagen was determined by initial treatment with a highly purified preparation of collagenase (Advanced Biofactors) which is free from protease contamination. The reaction mixture after incubation with collagenase was treated with 5% trichloroacetic acid. Radioactivity which remained in the supernatant was considered to be collagenase degradable protein. A blank was always carried out to which no collagenase was added, but which was otherwise treated under the same conditions. Radioactivity which was precipitated with trichloroacetic acid was taken as a measure of non-collagen protein synthesis. Prolylhydroxylase was measured according to the method of Hutton et al. (1966) using chick embryo substrate. (We are grateful to Dr. George Fuller, University of Rhode Island for giving us the substrate). The reaction was done in half the original volume (0.5 ml). After the reaction, tritiated water was separated from the reaction mixture by passing through a 1 ml Dowex 50 x 4H⁺ column and subsequent treatment with charcoal. The background counts were 60 C.P.M. Hydroxyproline in the tissues was determined after acid hydrolysis by the method of Neuman and Logan (1950). ^{14}C -hydroxyproline was determined by a method described by Peterkofsky and Prockop (1962). Protein was determined by the method of Lowry et al. (1951).

RESULTS AND DISCUSSION

Effect of Paraquat on Collagen Synthesis in the Lungs of Adult Rats

Table 1 shows the effect of paraquat treatment on collagen synthesis in adult rat lungs. Prolylhydroxylase activity, which is a marker

of collagen synthesis, has been significantly elevated. However, the total hydroxyproline content, which is an indicator of total collagen content in the lungs of the treated and control animals, was almost the same. There was also no difference in the incorporation of ^{14}C -proline into ^{14}C -hydroxyproline and into non-collagenous protein in the lungs of the paraquat treated and control animals.

Previous studies have produced conflicting data on the effects of paraquat treatment on collagen synthesis in rat lungs. Hollinger and Chvapil (1977) reported that there is an increase of prolylhydroxylase activity in rat lungs seven days after acute paraquat treatment but no increase in total collagen content. However, further studies were not in agreement with this finding and indicated that paraquat is not a good model for experimentally induced lung fibrosis (Hollinger et al, 1978). This conclusion was also supported by another group of workers (Autor and Schmitt, 1977). However, other reports indicate that paraquat produces substantial increases in prolylhydroxylase activity and collagen synthesis after a few days of treatment (Thompson and Patrick, 1978; Greenberg et al., 1978a & b). There is much less data on the effects of paraquat treatment on the total collagen content of the lungs. Although a small increase in the total hydroxyproline content of the lungs of paraquat treated animals was observed at 21 days after an acute dose, no change was seen at seven days (Hollinger and Chvapil, 1977). Our data indicates that there is a significant increase of prolylhydroxylase activity in the lungs of paraquat treated animals nine days after an acute dose (25 mg/Kg). However, we could not demonstrate any increase in total hydroxyproline content or increased hydroxyproline synthesis in the lungs of these animals. These results might be explained by a rapid

TABLE 1. Collagen synthesis in lung slices of normal and paraquat treated rats.

	Hydroxy-proline (ug/am tissue)	Prolyl-hydroxylase (DPM/mg protein)	Incorporation of ¹⁴ C-Proline Collagen-HP (DPM/mg protein)	Incorporation of ¹⁴ C-Proline Non-Collagen (DPM/mg protein)
Control Rat Lung	1316 ± 249	10360 ± 788	284 ± 66	5131 ± 1175
Paraquat Treated Rat Lung	1189 ± 309	36330* ± 3693	227 ± 32	4010 ± 1025

p < 0.01

The values are the mean ± standard errors of five separate determinations in each group. The significance of the results is calculated using Students t-test. The values which are significant are indicated by a asterisk (*).

degradation of collagen which is formed either in vivo or in vitro.

Bienkowski et al. (1978) have shown that a large portion of newly formed collagen is rapidly degraded in fibroblasts. It is possible that in a condition like paraquat toxicity, collagen degradation may be accelerated thus resulting in no net increase in lung collagen content. An alternate explanation may be that because of the presence of paraquat in the lung tissues there is an inhibition of collagen and protein synthesis. Paraquat induced lung fibrosis has been reported as an intra-alveolar fibrosis and large amounts of cellular debris and fibrin have been shown to accumulate in the alveolar space (Vijeyaratnam and Corrin, 1971). This may also explain why there is no significant increase in total collagen content in these tissues. In this case, prolylhydroxylase activity is not a good index of collagen synthetic activity.

Effect of Paraquat Treatment on Collagen Synthesis by the Kidney

We have also investigated collagen synthesis in the kidneys of rats treated with paraquat (Table 2). Unlike the results in the lungs, no

TABLE 2. Collagen synthesis in kidney slices of normal and paraquat treated rats.

	Hydroxy- proline (μ g/gm tissue)	Prolyl- hydroxylase (DPM/mg Protein)	^{14}C -Proline Incorporation Collagen-HP	Incorporation Non-Collagen
Control	381 \pm 87	2555 \pm 636	71 \pm 6	922 \pm 79
Paraquat	393 \pm 40	1581 \pm 428	79 \pm 4	1220 \pm 53

p < .05

The values are the mean \pm standard error of five separate determinations in each group. The significance of the results is calculated using Students t-test. The values which are significant are indicated by an asterisk (*).

difference in the specific activity of prolyl hydroxylase in the treated and control rats was seen. There was also no difference in the total hydroxyproline content of the kidney or on collagen hydroxyproline synthesis. Morphological evidence suggests that there is kidney damage associated with many paraquat poisonings. Paraquat has been described as a "hit and run" type of toxic agent. After paraquat ingestion, nearly 80-90% of the compound is excreted in the urine within 24 hrs. Hence, there is an increased total concentration of paraquat in the kidney during this period. Necrosis of proximal tubules has also been shown in kidneys affected by paraquat (Fowler and Brooks, 1971). The transient presence of high concentrations of paraquat in the kidney, however, did not result in increased prolylhydroxylase activity as was the case for the lung where paraquat is concentrated and may remain for longer periods of time.

Effect of Paraquat on Lung Tissues in Culture.

In order to find out whether there is any direct effect of paraquat

on collagen synthesis, paraquat was added to culture medium containing lung slices from young rats. Addition of paraquat markedly decreased prolyl-hydroxylase activity and collagen synthesis in these cultured tissues (Table 3). There was also a concomitant decrease in total protein synthesis. Although the values shown were obtained after 48 hrs of treatment, decreases in prolylhydroxylase activity were detected as soon as 3 hrs after treatment. Similar results were obtained with neonatal rat lungs and adult rat kidneys. Thus, paraquat produces an acute general toxicity in the organ culture system unlike that observed in vivo and does not have any specific direct effect on these tissues.

TABLE 3. Effect of paraquat addition on collagen synthesis of lung slices obtained from young rats.

	Prolyl-Hydroxylase	Collagen Synthesized	Protein Synthesized
Control	11838	5062	88689
Parquat (10 ⁻⁴ M)	5768	527	89821
Parquat (10 ⁻³ M)	3505	670	22304

All values are expressed as cpm/mg protein and are the mean of three determinations. Estimations were done 48 hrs after the addition of paraquat.

Effect of Parquat on Neonatal Lung Cells in Culture

Since the lung is composed of 40 different cell types and paraquat may have a specific effect on only some of these which would not be apparent in an organ culture system, we examined the effects of paraquat on cells proliferated by an explant technique and on those derived by trypsinization of lung tissue. We observed that cells proliferated by the explant technique are different from those obtained by the

trypsinization method. The cells obtained by the explant procedure were heterogeneous in nature and at least four kinds of cells were observed, small round cells, large ciliated cells, fibroblasts and epithelial cells. Cells obtained by the trypsinization method were round, non-ciliated, and smaller in size, and divided more rapidly than those derived from explants. Paraquat had different effects on both cell populations (Table 4). In cells obtained by the explant method, paraquat addition markedly decreased prolyl hydroxylase activity and collagen synthesis, similar to the effects seen in whole slice organ culture. However, addition of paraquat to cells obtained by the trypsinization method did not decrease prolyl hydroxylase activity but showed inhibition of collagen synthesis, indicating again that in the lung the effect of paraquat on the activity of this enzyme was not a consistent indicator of its effects on collagen synthesis.

The selective action of paraquat on these cell populations is interesting. It is possible that cells derived from the explant procedure may take up more paraquat than those obtained by trypsinization.

TABLE 4. Effect of addition of paraquat to the lung cells in culture.

	Prolyl Hydroxylase		Collagen Synthesis	
	Explant	Trypsin	Explant	Trypsin
Control	113878	37916	17041	6304
Paraquat ($10^{-4}M$)	43577	40794	13950	4269
Paraquat ($10^{-3}M$)	22105	43084	1182	2951

All values are expressed as CPM/mg protein. Determinations were done 48 hrs after the addition of paraquat.

thus contributing to their higher sensitivity to this agent. For example, it has been observed that macrophages are more sensitive to paraquat than fibroblasts because macrophages take up more paraquat than fibroblasts (Styles, 1974). Similarly prolyl hydroxylase and collagen synthetic activity may have different thresholds of sensitivity to paraquat in vitro, the latter being affected at concentrations which do not inhibit activity of this specific enzyme. Further investigation using specific established cell lines will be necessary to clarify whether the paraquat resistant cells obtained by trypsinization have any relation to the cells which proliferate in paraquat toxicity, namely, type II cells.

Effect of Paracuat on the Activation of Prolyl Hydroxylase

It has been shown in many cell lines in their early-log phase, that prolyl hydroxylase activity could be increased by the addition of small amounts of sodium ascorbate (Cardinale et al., 1975). In order to see whether paraquat has any effect on this activation process, lung cells from early log phase were treated with paraquat in the presence and absence of sodium ascorbate. As seen in Table 5, the specific activity of prolyl hydroxylase in the lung cells was increased by short incubation with sodium ascorbate. However, it was found that the addition of paraquat prevented the activation of prolyl hydroxylase induced by sodium ascorbate. Addition of paraquat alone decreased the specific activity of prolyl hydroxylase, to a lesser degree than the combination of paraquat and ascorbate.

Ascorbate is needed for collagen synthesis. This data indicates ascorbate is not active in the presence of paraquat. The significance

TABLE 5. Effect of paraquat on prolyl hydroxylase activation by sodium ascorbate in lung cells at early-log phase.

Condition	Prolyl Hydroxylase Activity (CPM/mg Protein)
Control Lung Cells	39481
Lung cells + Sodium Ascorbate $(10^{-4}M)$	72715
Lung cells + Paraquat $(10^{-4}M)$	32632
Lung cells + Paraquat $(10^{-4}M)$ + Sodium Ascorbate $(10^{-4}M)$	18577

Lung cells (early-log phase) which were obtained from explants were incubated in medium containing ascorbate, paraquat or both for a period of 3 hrs. The cells were then sonicated and assayed for prolyl hydroxylase.

of the apparent synergistic action of ascorbate in increasing paraquat inhibition of prolyl hydroxylase is not known at present. An attempt to prevent or reduce paraquat toxicity with sodium ascorbate in adult and neonatal rats in this lab was unsuccessful (Kuttan, unpublished observations).

The results presented here show that paraquat induces its damage by an acute toxic effect on lung cells and the resulting fibrosis does not biochemically resemble a process in which collagen deposition plays a major role. This finding is in agreement with morphological evidence which indicates that at ten days after an acute dose of paraquat similar to that used in these studies, the pulmonary fibrosis induced in the rat is typically cellular with the lung architecture being obliterated by a dense mass of fibroblastic tissue containing only small quantities of collagen (Smith and Heath, 1976). The observed increase in prolyl hydroxylase activity in vivo after paraquat administration appears specific to the lung and is a secondary event which does not correlate

well with the acute inhibition of this enzyme by paraquat in vitro or with collagen synthetic activity.

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COMPOSITION OF ALVEOLAR BASEMENT MEMBRANE

To obtain baseline data on the connective tissue components of normal lungs, basement membrane-containing lung extracellular matrix was obtained from several species (rat, rabbit, cow and dog) of various ages. The amino acid compositions of the preparations from all species tested were similar to each other and to extracellular matrix prepared similarly from other tissues such as the kidney and the retina. The results are presented in the following manuscript.

Kuttan, R., Spall, R.D., Duhamel, R.C., Sipes, I.G., Meezan, E. and Brendel, K. Preparation and composition of alveolar extracellular matrix and incorporated basement membrane. Submitted to Lung.

PREPARATION AND COMPOSITION OF ALVEOLAR
EXTRACELLULAR MATRIX AND INCORPORATED BASEMENT MEMBRANE

Running Head: Alveolar Extracellular Matrix

R. Kuttan¹, R. D. Spall, R. C. Duhamel,
I. G. Sipes, E. Meezan², K. Brendel

Departments of Pharmacology and Anesthesiology
University of Arizona Health Sciences Center
Tucson, Arizona 85724

¹Present Address: Department of Biochemistry, Connective Tissue Research
Laboratory, Baylor College of Medicine, Texas Medical
College, Houston, TX 77030

²Present Address: Department of Pharmacology, University of Alabama in
Birmingham, Birmingham, Alabama 35294

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Naval Research.

Send proofs and reprints to Dr. Meezan

ABSTRACT

A cell-free alveolar extracellular matrix has been prepared from the lungs of several species of young and adult animals by a procedure consisting of extraction of parenchymal fragments with the detergents Triton X-100 and sodium deoxycholate. The isolated extracellular matrix preparations were ultrastructurally heterogeneous consisting of amorphous basement membranes and associated interstitial collagen and microfibrillar components as shown by histochemical staining and their electron microscopic appearance. The amino acid and carbohydrate compositions of these preparations had a collagenous nature which resembled in many respects that of some ultrastructurally pure basement membranes. Urea extraction of extracellular matrix from adult animals solubilized a distinctly less collagenous fraction which was particularly rich in the acidic amino acids aspartic and glutamic acids and had a chemical similarity to acidic structural glycoproteins. In contrast, identical extraction of the matrix from fetal calf yielded a more collagenous extract possibly due to less crosslinking of the immature collagens. Extraction of adult samples with urea-mercaptoethanol and pepsin digestion of the insolubilized residues gave more collagenous fractions which appeared from their carbohydrate compositions to be enriched in basement membrane collagens. Gel electrophoresis of extracts of extracellular matrix samples from several species gave similar multicomponent patterns consisting of collagenous and noncollagenous polypeptides. Our ultrastructural and chemical examination of these alveolar extracellular matrix preparations clearly indicate that they cannot properly be designated as pure alveolar basement membranes. These findings and the anatomical characteristics of the alveolar blood-air interface make it probable that preparations isolated by others using sonication procedures, with chemical characteristics similar to those

INTRODUCTION

The alveolar-capillary barrier is the morphological and functional mediator of gas exchange in the lung. An integral component of this barrier is the alveolar basement membrane which lies between the capillary endothelium and alveolar epithelium and serves as a supporting structure upon which these cells rest, as well as a boundary layer which defines the separation between the blood and air spaces of the lung. [18,30]. The juxtaposition of the alveolar basement membrane between two different cell types and its morphological features by which it appears alternately as a double layered structure separated by an intervening interstitial space, or as a single layer which appears to be the fusion of two membranes which may be heterogeneous in nature [12] make it a complex structure not easily accessible to isolation and biochemical investigation. For this reason it has been one of the least studied basement membranes in regard to chemical composition and structure.

Kefalides and Denduchis [15] reported on the composition of an alveolar basement membrane fraction obtained from dog lung which was prepared by a combination of exhaustive extraction of thin sections of peripheral lung tissue with 0.3M acetic acid at 4°C and sonication in physiological saline.

Bray and LeRoy [1] isolated an alveolar basement membrane preparation from human lungs using a sieving and sonication procedure analogous to that previously employed for the preparation of bovine glomerular basement membrane [29]. Although both of these preparations had a collagenous composition similar to those obtained from the morphologically pure and chemically well characterized basement membrane of the lens capsule and renal glomerulus, no electron microscopic examination of their structure was given to reveal the

reported here, were not ultrastructurally pure alveolar basement membranes as reported but were actually heterogeneous mixtures of basement membranes, interstitial collagens and microfibrils. It would appear that the alveolar basement membrane cannot be isolated in a form comparable to ultrastructurally pure basement membranes such as that of the renal glomerulus.

Key Words:

Lung extracellular matrix
alveolar extracellular matrix
alveolar basement membrane
lung basement membrane
lung collagen

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lung basement membrane
lung collagen

possible extent of contamination by other connective tissue components. In view of the known intimate association of the basement membranes of the alveolar wall with striated interstitial collagen and microfibrillary components as revealed by several electron microscopic examinations of lung tissue [18,19,28], it was difficult to see how simple sieving, sonication and acid extraction procedures could result in the isolation of a basement membrane fraction substantially free of these components. Madri and Furthmayr [20] have recently demonstrated the collagen polymorphism of the lung by immunochemical techniques and have shown that lung parenchyma contains at least four distinct types of collagen (I, III, IV and V (AB_2)).

We have therefore isolated alveolar extracellular matrix from the lungs of several species of animals of different ages using a detergent extraction procedure which has proved valuable in the isolation of ultrastructurally pure and intact basement membranes from renal glomeruli and tubules and retinal and brain microvessels [5,21,22], as well as in characterizing the more complex extracellular matrix obtained from human retina, brain, peripheral nerve and placenta [4,23-26]. Our results indicate that detergent extraction of lung parenchymal tissue yields an extracellular matrix consisting of amorphous basement membrane material as well as enclosed and associated interstitial collagen fibers and microfibrils, which although having a chemical similarity to ultrastructurally pure basement membranes, are morphologically more similar to the ultrastructurally heterogeneous extracellular matrix previously characterized from human placenta [24,25]. Selective extraction of the alveolar extracellular matrix with urea solutions under reducing and nonreducing conditions followed by pepsin treatment of the insoluble residue yielded markedly collagenous fractions and those which more closely resembled acidic structural glycoproteins [3,6] in their composition.

MATERIALS AND METHODS

Preparation of Alveolar Extracellular Matrix

Alveolar extracellular matrix was prepared from the lungs of several species of animals of different ages by a modification of a procedure developed in this laboratory for the isolation of basement membranes and extracellular matrix from other tissues and tissue fractions [4,5,21-25]. Lungs of dog, rabbit, adult rat and newborn rats were obtained fresh and processed on the same day. Fetal calf lung which was obtained frozen from Pelfreeze was processed as soon as it arrived. The parenchymatous material from the lung was separated from the large vessels using a blade at 4°C. Care was taken to exclude large airways, blood vessels and fibrous tissue. The rest of the isolation was done at room temperature (20°C). The tissue was washed well in isotonic saline and suspended in 20 times its volume of saline. It was homogenized using a Polytron (Brinkmann Instruments) at an intermediate setting for 30 sec for soft tissues and 2 min for tougher tissues. The homogenate was filtered through a 400 µm opening nylon sieve to remove large particles. These consisted mainly of blood vessels and portions of bronchi which are not homogenized to small fragments under the conditions used as are the more fragile alveoli. The filtrate was put through a 110 µm opening sieve and this filtrate was discarded. The material collected on the sieve was washed with saline and after examination by phase contrast microscopy to assure the absence of large airways and blood vessels was treated with a solution of 4% Triton X-100 in phosphate buffer (0.2 M, pH 8.0). The tissue weight to Triton volume ratio was usually 1:10. All extractions were done in the presence of a proteinase inhibitor (phenylmethylsulfonylfluoride 50 µg/ml), a bacteriostat (sodium azide 50 µg/ml) and a fungicide (caprylic acid 50 µg/ml). In order to further reduce the possibility of proteolysis, the Triton mixture was changed after 1/2 h, 2 h and 15 h. During each change the tissue was washed with saline. After 24 h the tissue was filtered and washed. It was

then stirred with sodium deoxycholate (4%). After 24 h of stirring, the tissue was filtered and washed first with 0.1% sodium bicarbonate solution, and then with distilled water. This material was then treated with deoxyribonuclease (100 µg/ml, 1 h, 37°C) in the presence of calcium and magnesium ions. When the amount of the tissue was large, this treatment was repeated. The material after this treatment was washed well in water and preserved with sodium azide.

Histology

Samples of extracellular matrix preparations were embedded for routine light microscopy and periodic-acid-Schiff reagent counterstained with hematoxylin was used to assess gross cellular contamination with every sample which was analyzed. Jones silver stain which specifically stains basement membrane material black was used to distinguish basement membrane materials. Interstitial collagen (Type I) was detected by staining with Van Gieson's solution which stains collagen red. In order to determine the extent of fibrin contamination, preparations were stained with phosphotungstic acid-hematoxylin which gives a blue color when fibrin is present.

Immunofluorescence Reaction

The antigen was a preparation of basement membrane purified from rabbit renal tubules by extraction with detergents as described previously [5,21,22]. Hyperimmune antiserum was raised in a young female goat by an initial injection of an emulsified suspension of tubular basement membrane in complete Freund's adjuvant followed by booster injections at monthly intervals in incomplete adjuvant. An indirect immunofluorescence procedure was performed on cryostat sections (5 µm) of lung tissue or on basement membrane suspensions which were dried onto glass slides. The slides were incubated with antiserum for 30 min at room temperature. The goat antiserum was diluted 30 fold in 33%

(v/v) normal rabbit serum in buffered saline. The control serum was a pre-immune serum from the same goat used in similar dilution. There was no detectable fluorescence with the control, pre-immune goat serum diluted 30 fold in 33% rabbit serum as was the antiserum nor when diluted only 5 fold. After incubation with the antiserum or the control serum, the slides were washed and incubated for 15 min with the secondary antiserum, fluorescein-conjugated rabbit antigoat IgG purchased from Miles Laboratories, Inc. After washing with phosphate buffered saline, the slides were mounted in buffered glycerol and examined with a Zeiss microscope equipped with epiillumination and filters for fluorescence detection.

Electron Microscopy

The alveolar extracellular matrix preparations were examined by electron microscopy. Matrix preparations were washed exhaustively with water and fixed in cold (4°C) Karnovsky's solution followed by washing and storage in 0.15 M, pH 7.4 cacodylate buffer. After post fixation in 0.5% osmium tetroxide solution, the samples were prepared for transmission electron microscopy. Ultrathin sections were stained with uranylacetate or bismuth subnitrate and examined on a Hitachi HS-7 transmission electron microscope.

Extraction of Alveolar Extracellular Matrix

A portion of the alveolar extracellular matrix was dried in a lyophilizer and 20 mg of this dried material was first extracted with 8 M urea in phosphate buffer (0.1 M, pH 7.7) for 24 hours at room temperature to solubilize non-covalently bound non-collagenous proteins and non-crosslinked collagens. After centrifugation of the extracted material, the residue was extracted with 8 M urea containing 3% mercaptoethanol to solubilize any proteins linked to the matrix by disulfide bonds. The remaining insoluble residue was washed with 0.5 M acetic acid and digested with pepsin (100 µg/ml,

4°C) in acetic acid for 24 hours to solubilize crosslinked collagens. The pepsin digest was neutralized with 10 N NaOH and then dialyzed against phosphate buffer (0.1 M, pH 7.2).

Amino Acid and Carbohydrate Analysis

Samples for amino acid analysis were hydrolyzed at 110°C for 22 h in constant boiling HCl under nitrogen. Samples were dried at room temperature over NaOH in a desicator and analyzed on a Beckman 121 C amino acid analyzer using a modification of the method of Guire, et al [9].

Carbohydrate analysis was performed by a gas chromatographic procedure modified from the method of Grimes and Greegor [8]. Samples were hydrolyzed in 2 N trifluoroacetic acid for 6 hrs at 110°C in sealed tubes using arabitol as an internal standard, followed by deamination, reduction and acetylation of the released sugars as described previously [25]. Analysis of the derivitized samples was carried out on a Hewlett-Packard automated gas-liquid chromatograph using an OV-225 column and an automatic integrator for the quantitation of peaks.

Gel Electrophoresis

Gel electrophoresis was done by the method of Furthmayr and Timpl [7] using 5% acrylamide gels in phosphate buffer (0.1 M, pH 7.2) with accompanying internal standards of type I collagen. Gels were stained with Coomassie Blue R-250 (0.025%) and destained in acetic acid (7%).

Materials

All chemicals were analytical reagent grade. The following chemicals were obtained from the respective manufacturers: Deoxycholate (sodium salt) from Sigma, U.S.A., Triton X-100 from Rohm and Haas, deoxyribonuclease (pancreatic) from Sigma, pepsin (twice crystallized) from Worthington and

collagenase type III from Advanced Biofactors. Fetal calf lung (frozen) was obtained from Pelfreeze.

Results

Isolation Procedures

The scheme for preparing and characterizing alveolar extracellular matrix is outlined in Figure 1. After removal of large blood vessels, bronchi and other unwanted connective tissue of the lung, the parenchymatous tissue was disrupted in a Polytron homogenizer for different times depending upon whether it was derived from fetal or adult lungs. Initial filtration through a nylon sieve with 400 μm openings served to remove large undisrupted tissue fragments consisting mainly of remaining blood vessels and bronchi which were discarded. The filtrate consisting mainly of alveolar fragments and smaller cell debris was passed through a 110 μm nylon sieve which retained most of the alveolar pieces and let the cell debris pass through to be discarded. The retained alveolar fragments were freed of cellular material in a graded fashion by stepwise treatment with Triton X-100, sodium deoxycholate and pancreatic deoxyribonuclease yielding a cell-free alveolar extracellular matrix as shown by periodic acid-Schiff hematoxylin staining of samples (Fig. 2). Analyses of the matrix for DNA and phosphate gave minimal values (<0.2% dry weight) comparable to those obtained for glomerular and tubular basement membranes isolated by an analogous detergent procedure [17]. Jones silver staining of this material yielded a pattern of black staining indicative of the presence of basement membranes (not shown). Van Gieson's stain gave both blue and light pink staining indicating the presence of basement membrane and associated collagens. Phosphotungstic acid-hematoxylin stain which was used to monitor fibrin contamination showed that these samples do not have any contamination from fibrin.

Although free of cells, these preparations probably contain a mixture of

interstitial collagens, microfibrillar components and elastin in addition to alveolar basement membrane, as indicated by their appearance in transmission electron microscopy (Fig. 3).

Isolated fragments of newborn rat alveolar basement membrane-containing extracellular matrix exhibited substantial fluorescence in an indirect immunofluorescent procedure using a goat antiserum raised against rabbit tubular basement membrane (Fig. 4a). Reaction of the antiserum with cryostat sections of intact fetal rabbit lung tissue results in marked fluorescence of the alveolar wall (Fig. 4b). In this case, a control slide using pre-immune goat serum showed no detectable fluorescence (not shown).

Chemical Analyses

The amino acid analyses of alveolar extracellular matrix prepared from the lung parenchyma of dog, rabbit, fetal and adult cow, and newborn and adult rat are shown in Table 1. The compositions from all species are typically collagenous in nature with a high content of glycine, proline, and alanine and appreciable amounts of 4-hydroxyproline and hydroxylysine. Sulfur-containing and aromatic amino acids were low in all samples and there was a virtual absence of the 3-isomer of hydroxyproline. In comparison with the matrix from adult animals, that from fetal or newborn animals showed a lower content of many of the amino acids abundant in collagen, glycine, proline, alanine, and hydroxyproline and a higher content of the acidic amino acids, glutamic and aspartic acid, the hydroxylated amino acids serine and threonine and the basic amino acids lysine and histidine. Enough samples were not analyzed, however, to allow a quantitative statistical analysis of these results.

Carbohydrate analyses of the preparations (Table 2) indicated that galactose and glucose were the most abundant sugars in each sample with lesser amounts of mannose and minor amounts of fucose. In each case the galactose

content was somewhat in excess of that found for glucose. Fetal and newborn animals showed lower amounts of glucose and galactose in the extracellular matrix than adult animals, but somewhat higher levels of mannose and fucose, although the limited number of samples analyzed did not allow a statistical comparison of these results.

Selective Extraction of Alveolar Extracellular Matrix

Initial extraction of alveolar extracellular matrix from fetal calf or adult cow lungs with 8 M urea alone solubilized about one-third of the matrix. Further extraction of the residue with 8 M urea in the presence of 3% mercaptoethanol solubilized another 40% of the material and controlled digestion of the final residue with pepsin at 4°C brought another one-sixth into solution leaving about 10-15% of the material intractable to denaturing solvents or enzyme digestion. Similar results were obtained by analogous extraction and pepsin treatment of the extracellular matrix from adult rat lung alveoli.

Amino acid analyses of the urea solubilized fractions obtained under non-reducing and reducing conditions and of the pepsin solubilized fractions are given for fetal calf and bovine lung alveolar extracellular matrix in Table 3. While the urea solubilized fraction from fetal calf lung has a composition which is markedly collagenous in nature and more collagenous than the unextracted matrix, the comparable fraction from bovine lung is distinctly less collagenous than the starting material. The fraction solubilized by urea-mercaptoethanol from bovine lung is more collagenous than that obtained with urea alone, while the analogous fraction from fetal calf lung is less collagenous than the urea fraction obtained under non-reducing conditions. Pepsin digestion of the residues remaining after urea-mercaptoethanol

extraction of fetal calf or bovine lung alveolar extracellular matrix liberated markedly collagenous materials in both cases as can be seen by the high content of hydroxyproline, glycine, proline and alanine in these fractions. The least collagenous fraction obtained in this series of extractions was that solubilized by urea alone from bovine lung alveolar extracellular matrix. This fraction was notable for its markedly lower content of glycine, hydroxyproline, proline and hydroxylysine and its enrichment in the acidic amino acids glutamic and aspartic acid and the hydroxylated amino acids threonine and serine.

Amino acid analyses of the extracts obtained from adult rat alveolar extracellular matrix (Table 4) yielded a pattern analogous to that seen for the comparable fractions from adult bovine lung (Table 3). With both tissues the urea extracts were the least collagenous and the richest in the acidic and hydroxylated amino acids and the pepsin-liberated materials most resembled collagen in their amino acid compositions.

Comparison of the carbohydrate compositions of the fractions obtained by selective extraction of the extracellular matrix from adult rat lungs (Table 4) revealed the urea-mercaptoethanol and the pepsin-derived fractions to be the richest in their content of the neutral sugars glucose and galactose while the urea-extracted fraction was lowest in the content of these sugars and higher in its content of mannose and the amino sugars. Only qualitative comparisons of these amino acid and carbohydrate patterns were attempted since variations in extent of extractability and the limited number of samples analyzed did not allow for statistically quantitative comparisons of these results.

SDS Gel Electrophoresis

The SDS-polyacrylamide gel electrophoretic patterns of the urea,

urea-mercaptoethanol and pepsin-derived fractions of the alveolar extracellular matrix from the lungs of several species are shown in Figure 5. The patterns are similar for each species. The urea extract of the alveolar extracellular matrix gave two bands near the α position of collagen. Although the nature of these bands has not been fully studied, preliminary evidence in the case of the fetal calf urea extracts indicates that these bands do not correspond in their mobility with the $\alpha_1(I)$ and α_2 bands of type I collagen, but instead have mobilities which resemble that of procollagen chains. The top band has been found to be partially digestible with pepsin (Kuttan, et al., unpublished observation). The nature of these bands in other species has not been studied. It is interesting to note here that the adult rat gave only one prominent band at the α position. There were also other bands in the urea extracts. The remarkable ones are the fast moving bands that appear after reduction, and the bands between the α and β chains. A large portion of the applied material did not enter the gel until reduced, a behavior typical of this type of sample [20]. The urea-mercaptoethanol extracts of several alveolar extracellular matrix preparations also gave bands at the α position. There were very strong bands between the α and β chains. There were some differences in the mobility of these bands in different species. Pepsin extracts yielded two bands at the α position and a band between the α and β position. The nature of these bands has not been investigated, but they are presumed to be collagenous in nature. Interpretation of these results is complicated by uncertainties about the pepsin susceptibility of matrix residues previously extracted with denaturing solvents (urea and SDS), and the difficulty of comparing the mobility of bands from extracted non-enzymatically treated samples to standards obtained by controlled pepsin digestion.

DISCUSSION

In this report we describe the isolation of alveolar extracellular matrix from the lungs of several species of young and adult animals. Although alveolar basement membrane is a principal component of this extracellular matrix preparation, the preparation did not consist solely of a morphologically-homogeneous, amorphous boundary membrane as is the case for the basement membranes obtained by analogous treatment of renal glomeruli and tubules, and brain and retinal microvessels [5,17,21,22]. Whereas detergent treatment [5,17,21,22] or sonication, and differential centrifugation [29] of renal glomeruli or tubules yield a preparation in which basement membranes devoid of interstitial striated collagen fibers, elastin or fibrin comprise the entire extracellular matrix of these organ subfractions, similar treatment of placental villi [24,25] or lung alveoli yield preparations which, although having compositional and immunological similarities to well characterized basement membranes, are ultrastructurally heterogeneous.

Several electron microscopic examinations of the alveolar-capillary barrier have shown it to contain numerous collagen fibers which appear to be incorporated with the basement membrane between alveoli and capillary lumina [18,19,28]. The intimate structural association of microfibrillar components of the extracellular space with the basement membranes of the alveolar wall have also been described in detail [19]. Therefore, in the absence of a careful morphological analysis of the isolated sample, one should be cautious in describing a preparation as that of alveolar basement membrane. In fact, of the two preparations previously described denoted as alveolar basement membrane, one was subjected to exhaustive extraction with acetic acid at 4°C in order to remove contaminating interstitial collagen [15], while the other showed evidence of contamination with elastin and had a chemical

composition markedly different from that of glomerular basement membrane isolated by an analogous procedure [1]. No electron microscopic evidence of the ultrastructural purity of either preparation was presented.

We have previously characterized, both morphologically and chemically, an extracellular matrix preparation from human placental villi, and shown that although the amino acid composition of this material bore a strong resemblance to that of human glomerular basement membrane, its electron microscopic appearance was that of amorphous basement membranes associated with interstitial collagen fibers and scattered patches of fibrin [24,25]. The preparation of alveolar extracellular matrix described in this paper bears a superficial resemblance to ultrastructurally pure basement membranes. Although there are similarities in the amino acid compositions, there are no distinctive chemical characteristics by which one can unequivocally label this preparation as alveolar basement membrane and ultrastructural examination clearly precludes this label. The amino acid compositions of our alveolar extracellular matrix preparations from cow and adult rat lungs are similar to that of the human alveolar basement membrane preparation reported by Bray and LeRoy [1], and it is probable that these are comparable materials prepared by detergent extraction and sonication, respectively.

The carbohydrate compositions of the samples of alveolar extracellular matrix examined also have a pattern similar to that seen in pure basement membranes, being richest in glucose and galactose, which are present in nearly equimolar quantities, with lesser amounts of mannose and traces of fucose [5, 16,21,22,29]. Similar proportions of these carbohydrates were present in the alveolar preparation of Kefalides and Denduchis [15]. The glucose content of the preparations is probably a good index of their collagenous content in general and their content of basement membrane collagens in

particular, since this sugar is not a common constituent of glycoproteins and is present in appreciable amounts only in type II collagen [10] and collagens of basement membrane origin [16,29]. Since in the lung, Type II collagen has only been reported as a constituent of trachea [10], this would not be a likely source of the glucose in our preparations.

The increased collagen-like profile of the amino acid compositions of alveolar extracellular matrix preparations from adult rat and cow compared with those from their fetal and newborn counterparts are similar to the age related changes in amino acid composition reported in rat glomerular basement membrane [11,14], and may reflect an increased deposition or reduced degradation of the collagenous components of the matrix with age. Sequential extraction, with urea under non-reducing and reducing conditions, of the alveolar extracellular matrix preparations from adult bovine lung and fetal calf lung also revealed an interesting age-related possible difference between the two preparations. While the urea extract of the adult sample was markedly less collagenous than the starting material, as was also seen for adult rat lung extracellular matrix and reported for glomerular basement membrane [13], the comparable extract of the fetal sample was highly collagenous in composition. This probably reflects a greater degree of non-crosslinked collagens in the fetal than in the mature matrix. The urea extracts of the samples from adult bovine and rat lungs were also notable for their increased content of the acidic amino acids, glutamic and aspartic acids, the polar amino acids, threonine and serine, and the neutral amino acids, leucine and isoleucine. In this respect their composition is similar to that of acidic structural glycoproteins isolated from lung parenchyma by extraction with urea-sodium borohydride [6] or acetic acid [3], although in contrast to the latter, our extracts still contained some collagenous components as evidenced

by the continued presence of hydroxyproline and hydroxylysine. The urea-mercaptoethanol extracts of the alveolar matrix from adult bovine and rat lungs were decidedly more collagenous than the urea extracts alone, indicating that an appreciable amount of collagenous material was bound to the matrix by disulfide bonds. The material obtained from the extracted residues of both fetal and adult matrix samples by controlled pepsin digestion was almost completely collagenous and similar in amino acid composition to the insoluble residues obtained after complete extraction of glomerular basement membrane under comparable dissociating and reducing conditions [13,14].

Of particular interest in this collagenous material derived from the core of insoluble material in the matrix from adult rat lung is its relatively high content of glucose and galactose. The high content of these sugars in this sample makes it likely that basement membrane collagens are a significant component of the insoluble alveolar matrix core, possibly bound by non-reducible covalent crosslinks. A significant amount of the basement membrane collagen is also bound in the matrix by disulfide bonds and can be extracted with urea-mercaptoethanol as can be seen by the high glucose-galactose content of this fraction derived from rat alveolar extracellular matrix.

The multicomponent nature of the alveolar extracellular matrix preparations and their qualitative similarity from species to species is indicated by the patterns obtained after SDS gel electrophoresis of urea and urea-mercapto-ethanol extracts of the preparations. The results confirm the importance of reducible disulfide cross-links in the organization of the matrix and its comprisal of several polypeptide chains of differing molecular weights of collagenous and non-collagenous characteristics. A similar complex organization has been reported for both pure basement membranes such as that

of the renal glomerulus [4,15] and for placental chorionic villar extracellular matrix [24-26] which is a complex of connective tissue components of which the trophoblast basement membrane is just one part. The multiple banding patterns given by these extracts of lung extracellular matrix were reproducible and were the same whether matrix isolation was carried out in the presence or absence of proteolytic inhibitors, indicating that they were not the result of in vitro proteolysis during isolation.

Because of the likely impossibility of separating a truly ultrastructurally-pure alveolar basement membrane preparation from the collagenous and other connective tissue components with which it is intimately associated in the alveolar matrix, future work on this basement membrane will have to concentrate on the fractionation of components from the matrix and their identification as basement membrane constituents by comparison with analogous components obtained from ultrastructurally pure basement membranes such as those of lens capsule and renal glomeruli. Until then it would appear prudent not to designate the clearly ultrastructurally heterogeneous preparations, which are derived by sonication or detergent treatment of tissue subfractions such as those from lung or placenta, as 'basement membranes' [1,3], nor to ascribe components present in such preparations such as fibronectin [2] as being basement membrane constituents.

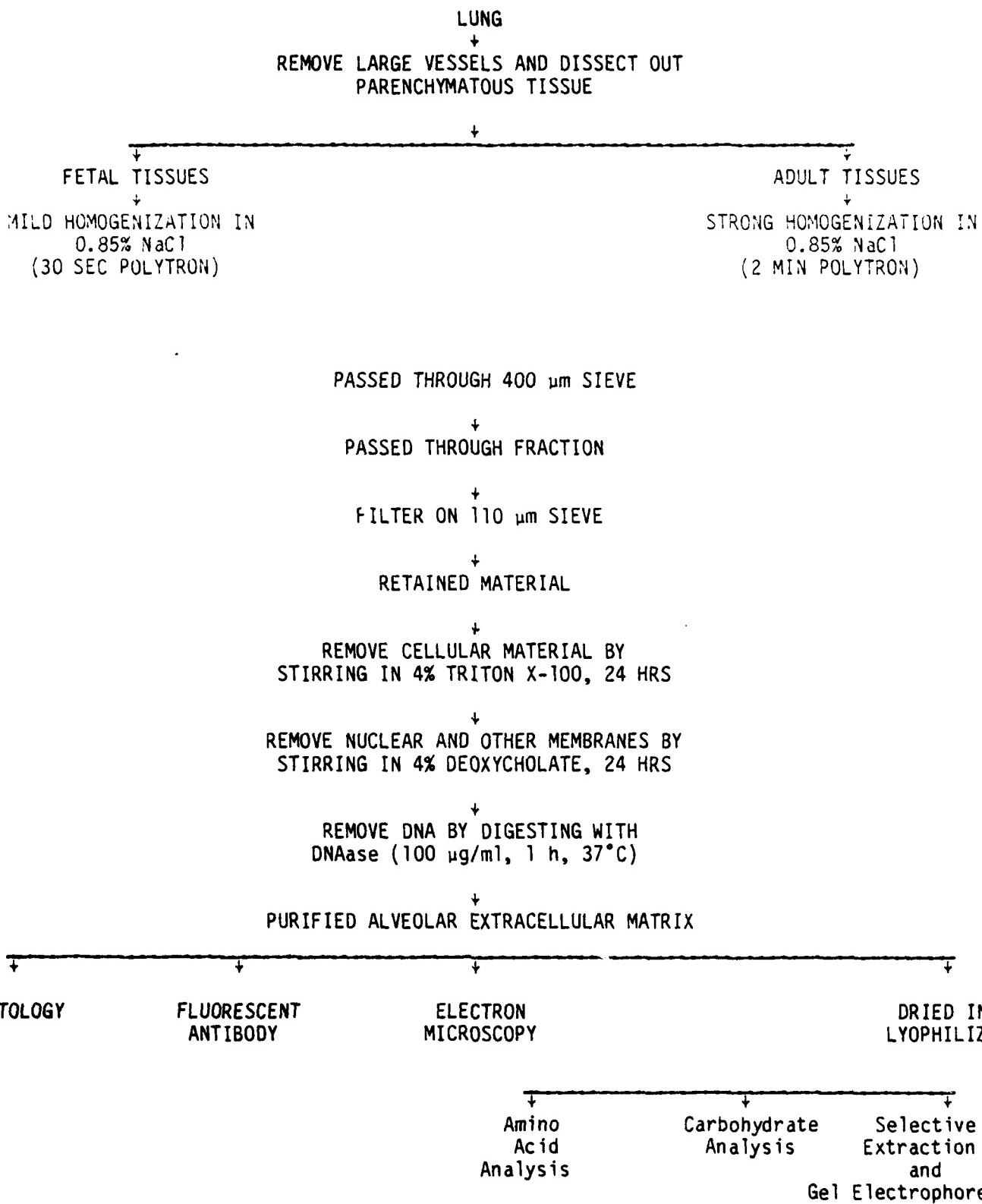


FIGURE 1

Figure 1. Scheme for preparation and characterization of lung alveolar extracellular matrix. All steps were performed in presence of 50 µg/ml phenylmethylsulfonylfluoride, sodium azide and caprylic acid. See text for details.

Figure 2. Light micrograph of section of isolated alveolar extracellular matrix stained with periodic acid-Schiff hematoxylin stain. Note absence of cells and staining of matrix (X 90).

Figure 3. Transmission electron micrograph of isolated alveolar extracellular matrix. Preparations consisted of amorphous basement membrane material, free and closely associated fibrils of interstitial collagen and other microfibrillar components (X 8250).

Figure 4A. Immunofluorescence reaction of newborn rat lung alveolar extracellular matrix with goat anti-rabbit renal basement membrane antiserum (X 90). Control goat serum at the same concentration produced no detectable fluorescence.

4B. Immunofluorescence reaction of cryostat section of fetal rabbit lung with renal basement membrane antibody (X 150). Control goat at the same concentration produced no detectable fluorescence.

Figure 5. SDS-polyacrylamide gel electrophoresis patterns of extracts from lung alveolar extracellular matrix preparations of various species.
1=Dog, 2=Rabbit, 3=Fetal Calf, 4=Bovine, 5=Adult Rat, 6>Newborn Rat.
A. Urea extracts, B. Urea extracts reduced with mercaptoethanol,
C. Urea-mercaptoethanol extracts, D. Pepsin extracts.

The mobilities of collagen chains derived from Type I collagen are as indicated.

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TABLE 1
Amino Acid Composition of Alveolar
Extracellular Matrix Preparations

Residues/1000 Amino Acids

<u>Amino Acids</u>	<u>Dog</u>	<u>Young Rabbit</u>	<u>Fetal Calf</u>	<u>Cow</u>	<u>New-born Rat</u>	<u>Adult Rat</u>
3-hydroxyproline	trace	trace	trace	trace	trace	trace
4-hydroxyproline	68.7	43.2	50.7	64.8	41.0	65.2
Aspartic Acid	42.2	54.3	59.0	48.2	49.0	52.1
Threonine	24.3	32.1	31.0	24.4	32.7	25.6
Serine	32.3	40.5	40.5	35.2	41.0	38.0
Glutamic Acid	62.7	75.7	83.3	72.1	69.5	68.4
Proline	101.2	89.7	88.0	95.4	87.8	98.9
Glycine	296.5	237.4	228.8	265.7	263.7	272.8
Alanine	130.1	117.5	107.0	120.3	124.8	118.1
1/2 Cystine	9.2	14.2	15.4	8.2	10.1	8.0
Valine	54.2	63.8	54.3	61.9	59.3	56.0
Methionine	8.3	10.6	11.1	8.7	6.1	7.2
Isoleucine	23.4	28.2	36.3	24.7	28.7	26.0
Leucine	42.0	59.9	53.3	50.2	59.3	51.4
Tyrosine	18.1	22.5	16.0	13.0	22.6	15.9
Phenylalanine	22.7	26.1	25.1	24.6	22.6	17.6
Hydroxylysine	10.7	7.7	7.9	9.4	8.2	7.7
Lysine	18.6	29.2	34.4	24.2	26.6	23.7
Histidine	8.1	11.9	11.7	8.5	10.1	9.5
Arginine	26.7	35.5	46.4	40.6	36.7	38.3

Duplicate samples of each preparation were analyzed except for adult rat where 4 samples were analyzed.

TABLE 2
Carbohydrate Composition of Alveolar
Extracellular Matrix Preparations

	<u>μg Sugar/mg Extracellular Matrix</u>					
	<u>Dog</u>	<u>Young Rabbit</u>	<u>Fetal Calf</u>	<u>Cow</u>	<u>New-born Rat</u>	<u>Adult Rat</u>
Fucose	0.55	0.85	0.93	0.68	0.83	0.71
Mannose	3.59	4.51	4.13	3.58	5.33	3.81
Galactose	18.03	10.81	11.88	14.37	12.03	12.39
Glucose	15.00	9.20	10.03	12.27	9.98	12.00

The results are the mean of duplicate samples from all species except for adult rat where 4 samples were analyzed.

TABLE 3
 Amino Acid Composition of Urea and Pepsin Extracts
 of Bovine Alveolar Extracellular Matrix
 Residues/1000 Amino Acids

	Fetal Calf Lung			Bovine Lung		
	Urea	Urea-ME	Pepsin	Urea	Urea-ME	Pepsin
3-hydroxyproline	2.7	trace	0.9	0	trace	3.8
4-hydroxyproline	82.9	58.5	85.4	23.2	53.0	56.0
Aspartic Acid	54.2	69.2	51.2	86.4	74.0	43.4
Threonine	23.3	32.5	21.4	43.5	38.1	23.0
Serine	40.6	47.0	41.1	54.6	53.0	32.5
Glutamic Acid	80.2	92.4	71.5	120.7	102.1	60.9
Proline	107.8	91.0	109.5	65.3	73.6	96.7
Glycine	290.2	235.0	295.6	146.3	202.7	287.5
Alanine	106.9	92.1	116.8	79.7	74.7	129.0
1/2 Cystine	2.7	9.8	1.8	13.2	17.3	4.9
Valine	30.9	43.4	41.1	53.6	48.3	74.6
Methionine	5.6	8.2	5.5	12.7	9.1	6.2
Isoleucine	16.1	25.6	20.1	38.6	30.8	25.7
Leucine	36.3	49.0	32.5	77.7	62.1	55.1
Tyrosine	8.3	12.7	7.9	19.6	17.8	14.2
Phenylalanine	18.6	24.9	18.3	32.8	30.1	31.6
Hydroxylysine	7.0	8.5	7.0	6.6	15.4	15.3
Lysine	28.5	33.6	24.7	46.2	34.8	12.4
Histidine	7.2	12.9	6.6	18.0	15.9	5.8
Arginine	50.2	53.6	41.3	62.0	47.2	21.7

The results are the means of duplicates from all samples.

TABLE 4
Amino Acid and Carbohydrate Composition
of Urea and Pepsin Extracts of Adult Rat
Alveolar Extracellular Matrix

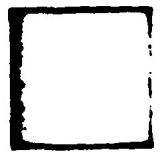
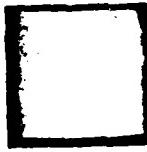
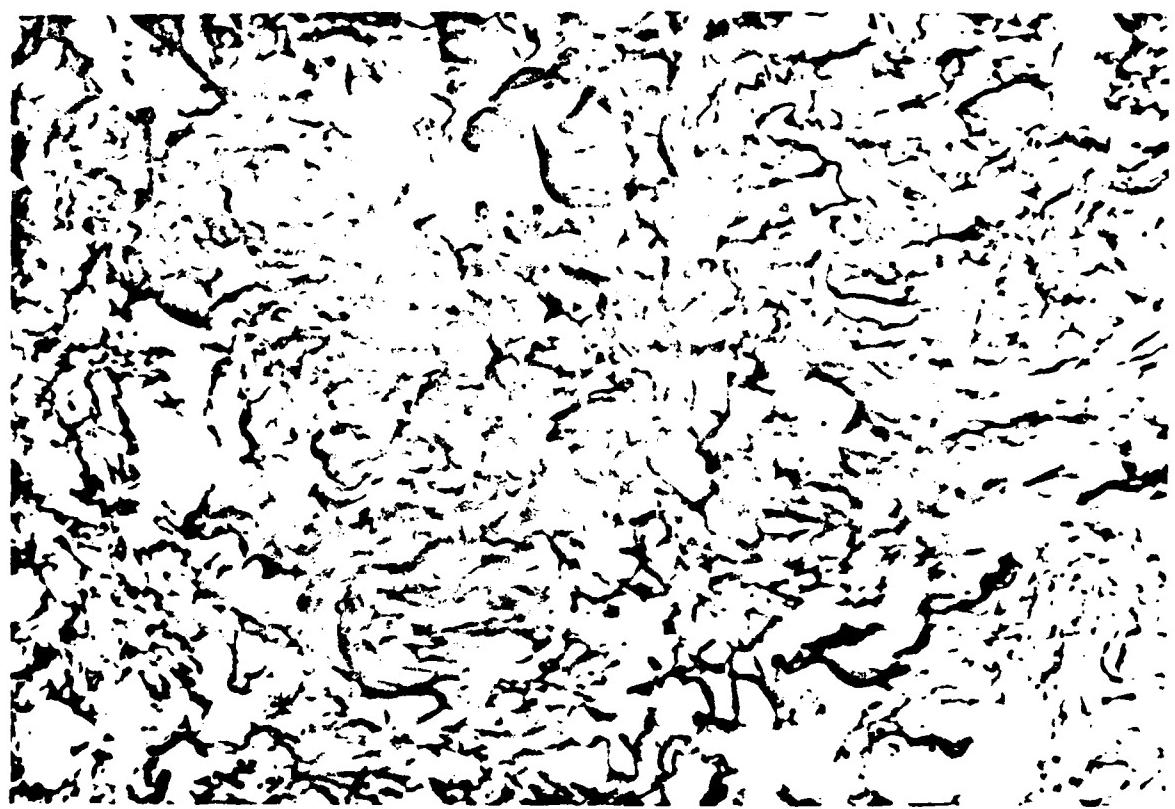
Residues/1000 Amino Acids

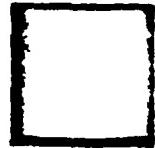
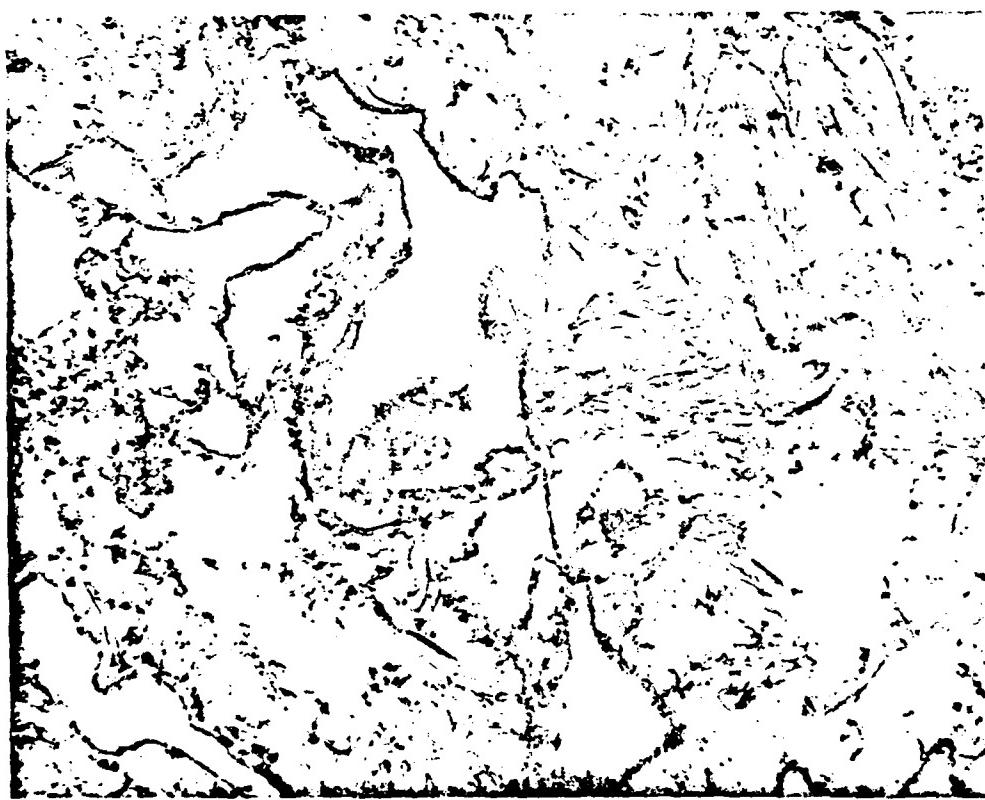
	Extra-cellular Matrix	Urea Extract	Urea-ME Extract	Pepsin
3-hydroxyproline	0	0	0	0
4-hydroxyproline	65.2	35.3	59.0	60.9
Aspartic Acid	52.1	85.8	77.9	60.5
Threonine	25.6	43.5	36.7	27.8
Serine	38.0	47.6	44.4	37.9
Glutamic Acid	68.4	98.1	96.9	50.3
Proline	98.9	71.1	78.7	104.3
Glycine	272.8	185.6	209.0	315.3
Alanine	118.1	78.8	85.2	100.5
1/2 Cystine	8.0	20.4	24.6	8.5
Valine	56.0	55.8	43.4	59.5
Methionine	7.2	10.7	12.4	5.2
Isoleucine	26.0	38.4	31.0	26.4
Leucine	51.4	68.3	57.3	50.8
Tyrosine	15.9	17.4	15.6	10.1
Phenylalanine	17.6	24.1	24.6	17.0
Hydroxylysine	7.7	8.3	18.6	12.7
Lysine	23.7	37.8	26.3	18.1
Histidine	9.5	12.3	15.0	5.9
Arginine	38.3	61.0	43.5	28.3

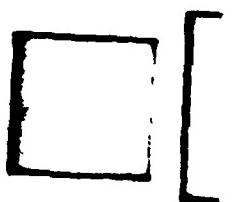
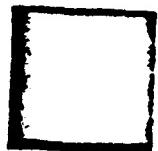
μg Sugar/mg Extracellular Matrix

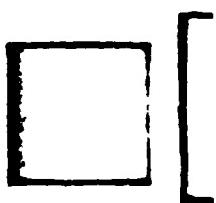
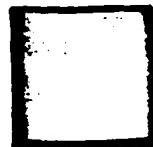
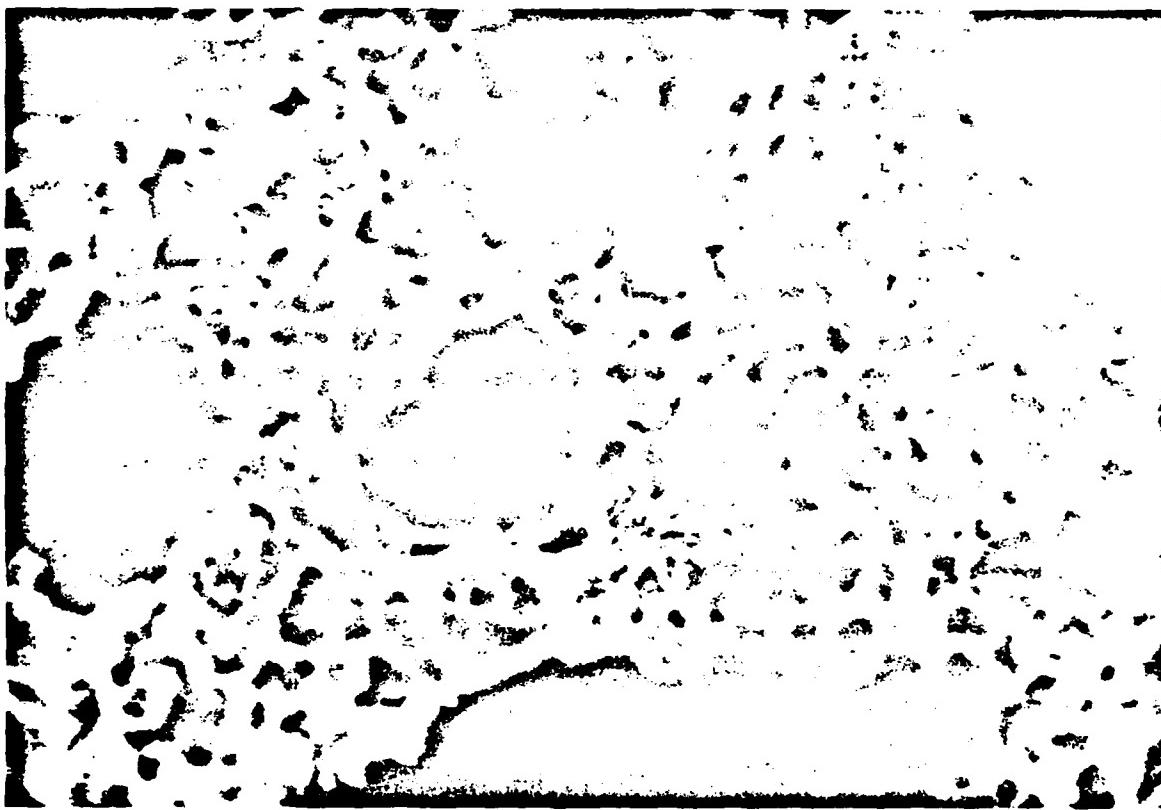
Fucose	1.60	1.28	1.20	1.07
Mannose	4.15	7.62	6.99	5.29
Galactose	11.94	11.11	24.71	19.66
Glucose	11.45	9.09	22.33	25.51
Glucosamine	4.13	12.77	10.44	6.56
Galactosamine	1.19	2.18	1.82	2.59

The results are the means of duplicates of all samples.

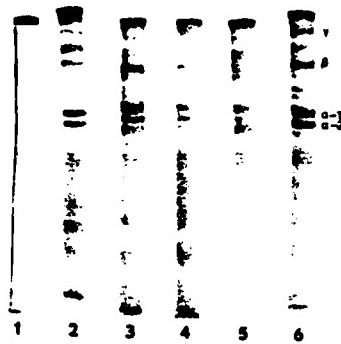




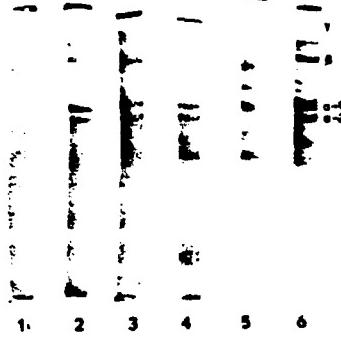




A Urea-Soluble (not reduced)



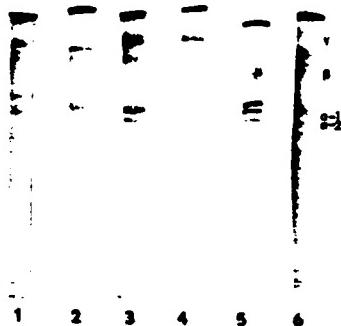
B Urea-Soluble (reduced)



C Urea, Mercaptoethanol-Soluble



D Papain-Solubilized



ISOLATION OF TYPE II PNEUMONOCYTES

In the past, methods to assess toxicity in the lung have used whole organ preparations or lung homogenates, but were relatively insensitive at detecting subtle biochemical alterations which may be present in different cell types. Therefore, it would be advantageous to conduct toxicity studies utilizing a homogeneous cell population from the lung. One particular cell type in the lung that has been of interest is the type II or alveolar septal cell. Pulmonary injury and repair from oxidative type insults have been shown to be mediated by these cells (1-4). A method was developed for the separation of pneumonocytes that is 50% faster than current methods and that has proven more reliable at removing macrophages than prior methods which rely upon size or density differences. The studies resulting in the development of the method and metabolic characterization of the cell preparation are described in the following manuscripts.

Lafranconi, W.M., Spall, R.D., Sipes, I.G., Duhamel, R.C., Meezan, E. and Brendel, K. A method for removal of macrophages to rapidly prepare lung cell fractions enriched in alveolar type II pneumonocytes. To be submitted to Experimental Lung Research.

Lafranconi, W.M., Brendel, K. and Sipes, I.G. (1981) Isolation and metabolic characteristics of lung cell types. Fed. Proc. FASEB (in press).

ABSTRACT

A method is described for the rapid preparation of lung cell fractions enriched in type II alveolar pneumonocytes. Isolated perfused rabbit lungs are exposed to Fe_3O_4 by tracheal lavage which permits pulmonary alveolar macrophages to phagocytize the particles. Alveolar epithelial cells are then selectively freed from the basement membrane matrix by critical placement of collagenase and elastase. Detached cells are harvested either by repeated tracheal lavage or by mincing the lobes and filtering freed cells through a series of nylon mesh sieves. Iron oxide containing macrophages are then removed from the harvested cells by a strong magnetic field. A final sizing of the macrophage depleted suspension yields a preparation enriched in alveolar type II cells. Eight million viable cells (95% type II) were obtained per rabbit lung when harvested by lavage, while 32×10^6 (88% type II) cells were obtained from mince lungs. These values for cell yield and relative purity are comparable to previously described separation methods which depend upon differences in cell density or size. A major advantage of the magnetic separation procedure is the substantially shortened preparation time, typically 2 hours instead of 4. The viability (90-95%), oxygen consumption (88 nmoles/ 10^6 cells/h) and incorporation of [^{14}C]acetate and [^{14}C]choline (0.44 and 0.115 nmoles/ 10^6 cells/h respectively) indicate these cells will be suitable for pharmacological or toxicological investigations.

A METHOD FOR REMOVAL OF MACROPHAGES TO RAPIDLY PREPARE LUNG
CELL FRACTIONS ENRICHED IN ALVEOLAR TYPE II PNEUMONOCYTES

W. Mark Lafranconi, Richard D. Spall, I. Glenn Sipes,
Raymond C. Duhamel, Elias Meezan* and Klaus Brendel

Department of Pharmacology
College of Medicine
University of Arizona
Tucson, Arizona 85724

*Department of Pharmacology
University of Alabama in Birmingham
Birmingham, Alabama 35294

Running Title: Preparation of Alveolar Type II Cells

Corresponding Author: Dr. Klaus Brendel
Department of Pharmacology
Arizona Health Sciences Center
Tucson, Arizona 85724
(602) 626-7729

INTRODUCTION

The lung presents a major target organ for toxic insult. It receives 100% of the cardiac output, and therefore maximum exposure to systemic toxins. Airborne toxins reach the lung through large volumes of inhaled materials which routinely pass over the delicate alveolar surfaces. Evaluation of specific pulmonary toxic responses have been difficult because of the extreme heterogeneity of cell types. Under toxic influences, these cells may change qualitatively, quantitatively or both. In the past, methods to assess toxicity in the lung have used whole organ preparations or lung homogenates, but were relatively insensitive at detecting subtle biochemical alterations which may be present in different cell types. Therefore, it is advantageous to conduct toxicity studies utilizing a homogeneous cell population from the lung to assess the specific biochemical events of toxic response.

One particular cell type in the lung that has been of interest to investigators is the type II or alveolar septal cell. Pulmonary injury and repair from oxidative type insults have been shown to be mediated by these cells (1-4). Further, type II cells produce pulmonary surfactant which is largely responsible for maintaining the low surface tension at the alveolar air-liquid interface (5-7). Alterations in surfactant production have been suggested in a number of disease states and in response to certain pulmonary toxins (8).

A number of procedures have been described for isolating type II cells from the lungs of both rats and rabbits (9-15). Most of these methods rely on density differences to separate cell types from a monodispersed lung digest. More recently procedures which use centrifugal elutriation for the separation

of cells on the basis of size have been described (12-16). The same disadvantage is apparent for both procedures. Type II cells have densities and sizes similar to macrophages and lymphocytes. Therefore, separation of cells based on size or density may very well be incomplete because of contamination from other cells of similar density or size.

Consequently a method to separate pneumonocytes based on techniques other than density centrifugation or elutriation was developed. It was reasoned that if macrophages could be induced to internalize magnetic particles they could be effectively removed from the suspension with a strong magnetic field. Other cell types could be removed from the lung preparation by perfusion and sizing techniques as well as by critical enzyme placement. These methods provide good yield, purity and reproducibility. Additionally, this method can be completed in less than two hours compared to over four hours required for other methods.

MATERIALS AND METHODS

Animals

White New Zealand male rabbits (2 Kg) were obtained from a local vendor (Blue Ribbon Ranch). Rabbits were fed standard Purina^R Rabbit Chow ad libitum and maintained in a temperature controlled room (22°C) with 12 hrs of light and 12 h of darkness in the University of Arizona Division of Animal Resources facilities.

Perfusion Medium

Perfusion medium was composed of 128 mM NaCl, 5 mM K₂SO₄, 5 mM KCl, 2.5 mM sodium phosphate buffer pH 7.4, 17 mM HEPES (n-2-hydroxyethyl piperazine n-2-ethane sulfonic acid), 5/5 mM glucose. This was adjusted to pH 7.4 with 2N HCl, (during later stages of cell harvesting this buffer was brought to 10% fetal calf serum with GIBCO F.C.S.). The buffer solution was prepared no more than 24 h in advance, filtered through 0.45 um milipore filters and oxygenation with 95% O₂, 5% CO₂ 20 minutes prior to use.

Magnetite Preparation

Magnetic iron oxide (magnetite) was freshly prepared for each experiment. A mixture of two ml of 250 mM FeSO₄ and 2 ml 250 mM FeCl₃ were added dropwise to 100 ml of boiling 0.2 N NaOH. A precipitate formed immediately and the mixture was allowed to boil 10 min. The precipitate was removed from the beaker by decanting the liquid phase and then resuspending the precipitate in 10 ml of distilled water. The suspension was transferred to 50 ml centrifuge tubes and centrifuged at 1000 x g. The supernatant was removed by suction and the precipitate washed three additional times in the same manner. The washed magnetite was then suspended in 10 ml of fetal calf serum (GIBCO)

and dialyzed overnight against distilled water. The dialysate was sonicated under cooling at 50% pulsation mode - 50% maximal output with a Sonifier model 350 (Branson Sonic Power Co.). The preparation was transferred to a 20 ml glass vial and kept at -20°C until used. NOTE: The colloidal iron oxide must not be allowed to come into contact with strong magnetic fields (e.g., magnetic stirrer) at any time as this will cause the particles to magnetize and aggregate the suspension.

Dispersing Enzymes

One hundred ml of dispersing enzyme solutions containing 0.5 mg/ml collagenase CLS type II (Worthington Biochemical Corp) and 11 ug/ml elastase (11 mg/ml Sigma) were freshly prepared for each experiment.

Preparation of Isolated Lungs and Instillation of Fe₃O₄

Animals were anesthetized with 50 mg/Kg sodium pentobarbital and treated with 2500 units/Kg sodium heparin by a single intraperitoneal injection. Fifteen min later, the ventral side of the animal was incised and the aorta transected. Both the trachea and the pulmonary artery were cannulated with 18 gage steel cannulas. After the cannulas were in place, the heart was trimmed away from pulmonary tissue. The pulmonary artery cannula was attached to an elevated 1 liter aspiration bottle containing perfusion medium and the system perfused until 1 liter of buffer was used. Total hydrostatic pressure was 1 meter of water.

To remove free macrophages, 20 ml of fresh buffer was instilled into the lung via the tracheal cannula. After 1 min, this buffer was removed and replaced with 25 ml of fresh buffer. The tracheal cannula was then closed with hemostats. The second instillation of buffer remained in the lung until after vascular perfusion.

Thus prepared, the lung system was removed from the rabbit en bloc and attached to a continuous perfusion apparatus through the pulmonary artery cannula. Warmed and oxygenated buffer was perfused continuously through the lung in a recirculatory manner at a flow of 35 ml/min. (See Fig. 1).

Once continuous perfusion began, hemostats were removed from the trachea and the intra-tracheal lavage fluid removed. With a 25 ml syringe, 20 ml of colloidal iron oxide were instilled into the lungs via the tracheal cannula. The perfusion buffer was replaced at this time with 100 ml of fresh buffer. After 20 min, the perfusion apparatus buffer was again replaced. The lung was then lavaged through the tracheal cannula three additional times with 20 ml of buffer each time. Lung prepared in this manner were then ready for enzymatic dispersion.

Perfusion with Collagenase and Elastase

Enzyme solutions (collagenase 0.5 mg/ml, elastase 1.1 mg%) were instilled into the lungs via the cannulated trachea. After instillation of collagenase and elastase, the trachea was closed with hemostats to prevent material from leaking back through the cannula. At this time, the buffer in the perfusion apparatus reservoir was replaced with fresh buffer containing enzymes, 3 mM CaCl_2 and 3 mM MgCl_2 , and lung perfusion continued through the pulmonary artery. In this way, proteolytic enzymes were supplied to the alveolar regions via tracheal instillation as well as to the endothelial side through perfusion medium via the pulmonary artery. Lungs were perfused with the enzymes for 10 min. This completed the first portion of the dispersion which was followed by either of the following two methods for harvesting cells.

Harvesting of Cells by Lavage

After enzymatic digestion, 100 ml of perfusate was removed through the trachea in small 10 ml aliquots. To avoid depletion of buffer, 10 ml of fresh

buffer were added to the perfusion apparatus reservoir when each 10 ml was removed from the lungs. After removing each sample, the lungs were gently massaged to free more cells. The material recovered in this way contained mainly alveolar epithelial cells. The recovered fluid was centrifuged at 50 x g for 10 min and the resulting pellet was resuspended in 2 ml of cold (4°C) fresh buffer supplemented with 10% fetal calf serum.

Harvesting of Cells by Mincing

The lung was removed from the perfusion apparatus after the 10 minute incubation with enzymes and placed on a 15 cm siliconized watch glass. Both cannulas were removed after the trachea and visible connective structures were cut away and the lungs minced with scissors until the pieces were approximately 0.5 cm in diameter. The pieces and fluid from mincing were transferred to a 250-ml siliconized Erlenmeyer flask and brought to 200-ml volume with HEPES buffer supplemented with 10% fetal calf serum (Gibco). The flasks were shaken for 10 min in a gyrotory shaker bath (New Brunswick model G-76) at a setting of 8 (approximately 100 rotations/min). The temperature was maintained at 37°C. Minces and free cells were consecutively passed through 180-um, 100-um, and 15-um nylon mesh screens (TETCO Inc., Elmsford, NY) and the filtrate collected in centrifuge tubes. The filtrate was centrifuged at 50 x g and the resulting pellet resuspended in 20 ml of cold (4°C) fresh buffer supplemented wth 10% fetal calf serum. After centrifuging, gentle vortexing of the pellet helped to resuspend the cells.

Removal of Macrophages by Magnetic Retention

The final steps in purification for both preparations 1 and 2 was conducted in the cold (4°C) and involved passing the cells through a strong magnetic field. To do this, a chamber was constructed with a narrow (2mm)

diameter. Cells flowed through the chamber at a rate of 1.5 ml/min with a linear velocity of 2 mm/sec. (See Figures 2 and 3). Effluent from the chamber contained a population of cells enriched in granular type II pneumonocytes. Cells thus collected were washed once in buffer, centrifuged at 100 x g, then resuspended in fresh buffer.

Cell Identification

Cells were identified by the modified Papanicolaou method on the basis of their characteristically dark staining inclusion bodies as described by Kikkawa and Yoneda (13). Cells were further identified by fluorescence microscopy according to the method of Mason and Williams (17) modified by using 1 ug/ml acridine orange instead of phosphine 3-R (18). With this method, type II cells fluoresce intensely yellowish green while other cells fluoresce diffusely green. For electron microscopic evaluation, cells were fixed for 3 h in 4% Karnovsky's fixative, then postfixed with 2% osmium tetroxide, dehydrated in ethanol, embedded in Spurr and thin-sectioned by standard techniques. Sections were stained with 5% uranyl acetate and were examined with a Hitachi model HS-75 electron microscope.

Centrifugal Elutriation

Centrifugal elutriation of the purified cells was performed in order to further characterize the cell population. Elutriation techniques described by Devereux et al (16) and Greenleaf et al (12) were used. Purified cell preparations were introduced into a Beckman JE-6 rotor on a 221-C centrifuge (Beckman Instruments, Inc., Palo Alto, Calif.). Rotor speed was maintained at 2000 rpm with a loading flow rate of 7 ml/min. Cells were eluted and 10 ml fractions collected at stepwise flow rates of 15, 22, 35 and 44 ml/min. The fraction collected at each flow rate was centrifuged at 100 x g for 5 min and

the pellet resuspended in 2 ml of buffer supplemented with 10% fetal calf serum. Cells in each fraction were identified by modified Papanicolaou staining, fluorescent microscopy with acridine orange, and by electron microscopy.

Oxygen Uptake

Oxygen uptake experiments were conducted with a Clark oxygen electrode with 5×10^6 cells suspended in glucose free HEPES buffer in a 5 ml chamber. Sodium succinate was added to a final concentration of 10 mM as described (19,12).

Lipogenesis Experiments

Radiolabeled precursor studies for lipogenesis were conducted with (^{14}C) acetate (2.0 uCi/uMole, New England Nuclear) and (^{14}C)choline chloride (5.37 uCi/uMole, New England Nuclear). Ten $\times 10^6$ cells in 2 ml buffer were incubated with 2 uMoles sodium acetate or 2 uMoles choline chloride for each time point and lipids extracted and washed according to the procedure of Folch et al (20). Saturated phospholipids were isolated from the lipid fraction according to the procedure of Mason et al (21) and submitted to scintillation counting. One hundred ul of using Aquasol as the scintillation fluid in a Beckman LS-330 liquid scintillation counter.

Viability

Cell viability was determined by trypan blue (0.20%) exclusion. Cells were counted on a standard hemocytometer.

RESULTS

Cell Yield and Purity

A summary of cell yield per rabbit is presented in Table 1. Large variations in yield were seen from one experiment to another which is reflected in the large standard deviations presented. It was found that the best cell recovery occurred in experiments in which initial pulmonary perfusion rapidly cleared blood elements from the lung. When clearance of blood elements was slow the recovery of cells decreased. The results of yield reported in Table 1 were compiled from 20 consecutive experiments and included data points from animals that did not perfuse rapidly.

Fig. 4 compares the relative purity and yield of cells obtained by lavage and minced lung methods. Lavage harvesting produced cells of higher purity but low cell numbers. Minced lung preparation yielded high cell numbers but lesser purity than lavage harvesting.

In both preparations, the high initial amounts of type II cells (55-68%) obtained indicate the enzyme preferentially releases these pneumonocytes from the lung matrix. This suggests that the infusion of enzymes in the perfused lung constitutes in itself an enrichment procedure since application of enzymes to the minced lung releases type II cells in initial concentrations of only 10-30% (data not shown) Other groups have reported preferential release of type II cells using trypsin (13,15).

Table 2 describes the nature of cellular contaminants in type II cell preparations. Counts were made of macrophages, ciliated epithelial cells, Clara cells, lymphocytes and eosinophils. Qualitatively, contamination by these cell types was similar in lavage or minced lung preparations; however,

contamination from the ciliated epithelial cells was significantly greater from minced lungs. This difference was consistent in each series of experiments.

Cell Viability

Evaluation of cell viability revealed that greater than 90% of the isolated cells from both procedures excluded trypan blue. Since exclusion of trypan blue is not the sole indicator of viability other means were also used to evaluate cells.

Oxygen Consumption

Oxygen consumption experiments were conducted on both isolated type II cells and the macrophages retained in the magnetic field. Ten mM Na succinate did not significantly alter the rate of O_2 consumption by cells when results were evaluated by the paired student t test. This indicated cell membranes were intact and impermeable to sodium succinate supporting the maintenance of good cell viability in the preparation (19).

Lipogenesis

The viability of cells were further evaluated by their ability to incorporate radiolabeled choline and acetate into the saturated lecithin components of chloroform-methanol extractable lipids. Fig. 6 shows the results from these experiments. Incorporation of both ^{14}C acetate and ^{14}C choline was linear for over two hours. Cells for the precursor studies were used immediately after purification without allowing time for recovery from the isolation procedure. A comparison of acetate and choline incorporation into macrophages was conducted as a control for the saturated lecithin assay. When expressed on a per cell basis, the incorporation of acetate and choline into saturated lecithins was greater in the type II cells.

Morphology

Isolated cells appeared normal upon examination by transmission electron microscopy. Mitochondria and cell membranes did not appear swollen or damaged. With minced lung harvesting a number of cells were observed which presented a slightly swollen appearance. All cells resembled those found in the intact lung.

Fractional Elutriation of Isolated Type II Cells

When cells were further separated on the elutriator, 86% of the recovered cells eluted at 16 or 24 ml/min flow rate. Ninety percent of the cells reoccurred at 24 ml/min were type II cells. Lesser amounts of type II cells were found at other flow rates as seen in Fig. 11. The use of elutriation helped confirm the identity of the cells isolated since the distribution of cells obtained at various flow rates correspond to distributions of type II cells reported by other groups (16,12).

Preparation Time

Isolation of cells according to the described procedure required less than two hours for completion. Preparation of the lung, including surgery, could be completed in 25 min while 20 min were required for perfusion and incubation after instillation of Fe_3O_4 . Enzymatic dispersion, mincing and shaking of the lung and sizing of freed cells were completed in 40 min. Twenty-five additional minutes were required to separate the cells magnetically and to wash the final cell preparation. Total time was 110 min.

DISCUSSION

These results show that a method for producing lung cell fractions enriched to greater than 85% purity for type II cells has been developed. This method can produce 32×10^6 type II cells per rabbit lung with similar values for viability (90%), oxygen consumption ($88 \text{ nMole}/10^6 \text{ cells/h}$) and lipogenic potential (0.115 and $0.44 \text{ nMole}/10^6 \text{ cells/h}$ choline and acetate incorporation respectively) as previously published isolation procedures. The one distinct advantage of this procedure is the rapid preparation time, typically 2 hours instead of 4 to 8 with other methods.

This method includes three basic techniques (i.e., critical enzyme placement, magnetic removal of macrophages and cell sizing through sieves) to remove contaminating cell fractions from mono-dispersed lung cell preparations without using time consuming differential gradient centrifugation. Past methods of isolating alveolar type II cells relied on density or size differences between cells to achieve separations (16,12,22). When large size or density differences exist between cells, these procedures afford good separation. In the lung however, type II and macrophage cell sizes and densities are too similar for complete separation. To overcome this problem, other investigators (13,15,22) developed procedures for type II pneumonocyte isolation based on addition of colloidally suspended dense materials such as BaSO_4 or fluorocarbon albumin which became phagocytized by the macrophages and increased their densities. Even though this manipulation of macrophages improved separation from other cell types, contamination still occurred.

The substitution of Fe_3O_4 for BaSO_4 or fluorocarbon albumin increases macrophage density but, in addition, it confers a unique property to

the cells prepared in the presence of colloidally suspended magnetic iron oxide (Fe_3O_4), in that macrophages containing iron oxide could be removed in the presence of a magnetic field. Electron microscopic examination of type II enriched fractions indicated that there was no contamination from macrophages which contained visible iron oxide. However, a small percentage of cells (5-10%) could be identified as macrophages which had not internalized Fe_3O_4 particles. It was assumed that these cells were non-viable at the time of iron oxide incubation or did not have access to the particles. Of the other contaminating cell types found in the effluent from the magnetic chamber, lymphocytes contribute most heavily and this contamination is persistent. Endothelial cells are partially removed early in the lung perfusion with collagenase and elastase, and appear in the circulating perfusate. A significant number of endothelial cells are also found on the 15 um filter. Perfusion of enzymes through the pulmonary artery provided discrete application of enzymes to the alveolar areas. Other contaminating cells (type I and ciliated epithelial cells) are also partially removed upon sieving or are destroyed during the dispersion procedure (23,24). They do not readily pass through the filter while smaller cells, including type II, can.

Throughout the cell separation procedures it was important to maintain cells in a mono-dispersed suspension. Clumping of cells interfered not only with the effectiveness of filtering and magnetic separation of cells but also reduced yields greatly. As a partial solution to this problem, separation procedures were conducted at $4^{\circ}C$, since cell aggregation has been shown to be a function of temperature (25). A further measure which reduced clumping was the addition of fetal calf serum to the buffer used during cell harvesting. The combination of separation in the cold and the addition of

protein to the buffers prevented most clumping and helped to improve cell yield.

The results of this study demonstrate the isolation of an alveolar type II cell preparation whose viability and purity as well as yield per animal were similar to those reported by other groups (9,12-16,22,26,27), but with the advantage of decreased preparation time. Staining with trypan blue was used as a gross screen for viability in all cell populations. Oxygen uptake experiments show that cells isolated with this procedure consume oxygen at a rate comparable to that reported in the literature (12,14,15,22) and radiolabeled lipid precursor experiments further demonstrated specific cellular lipogenic functions.

Other groups (12,15,28) reported cells required 30 minutes to 1 hour of incubation after isolation before linear uptake of radiolabeled precursors could be demonstrated. This incubation period was postulated as being necessary to allow cells to recover normal metabolic function after disruption by the isolation procedure. With the isolation procedure described in this report, linear uptake of radiolabeled precursors could be shown immediately after isolation of the cells eliminating the need for a lengthy recovery period. This may, in part, be due to the rapidity with which the cells were isolated. Other procedures require 3-8 h for separation and in that time some reversible metabolic alterations could occur. The method described in this report takes less than two hours to complete and does not require the cells to be subjected to lengthy centrifugations, thereby reducing the possibility of cell damage or other alterations.

Furthermore, differences in enzymes used for dispersing the pneumonocytes may be responsible for recovery time differences among procedures. Dobbs and

Mason (29) suggested alterations in membrane receptors may occur after treatment with trypsin. Further, Finkelstein and Mavis (30) reported release of NADPH cytochrome C reductase from intercellular membranes upon treatment with trypsin or elastase. Since our method primarily uses collagenase and elastase to remove cells from the extracellular matrix, it is conceivable that the cellular damage caused by these enzymes was different than methods which use trypsin (12,13,14,15,22) hence the decreased recovery time.

Disadvantages of this procedure obviously include its lack of generality; that is, while it might be useful in the isolation of type II epithelial cells, it does not provide adequate material for the isolation of type I or endothelial cell preparations.

The main potential advantage of this procedure is the fact that it takes less than 2 hours from sacrificing the animal to having an enriched type II cell suspension in a tissue culture media. Previously reported methods for isolating type II cells required 4-6 hrs of preparation time (9,12-16,22,26,27). It is conceivable that within this time frame metabolic alterations may be taking place which might contribute to cellular responses which differ from those in the intact animal. Numerous groups have reported biochemical and morphological alterations in type II cells in culture (9,11,26). Since it is not known what causes these alterations or even when and how they are initiated, it becomes important to isolate the cells rapidly.

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LEGENDS TO FIGURES

FIG. 1

Lungs were removed from anesthetized animals and attached to the recirculatory perfusion apparatus. Warmed and oxygenated buffer was continuously perfused through the isolated organ. Free macrophages were removed by lavage through the cannulated trachea. Colloidal Fe_3O_4 was introduced to the alveolar regions via the cannulated trachea.

FIG. 2

To separate cells the loading chamber and separating chamber were filled with buffer and air bubbles removed from the lines and chambers. Monodispersed cells were introduced into the loading chamber and the stopcock rotated to allow the pump to move fresh buffer through the loading chamber, carrying cells through the separating chamber and into the collecting tube.

FIG. 3

Enlarged figure of separating chamber: Monodispersed cells were introduced to the chamber and past baffles which reduced turbulent flow. Cells moved through the magnetic field with a linear velocity of 2 mm/sec which allowed retention of Fe_3O_4 containing cells while moving non-magnetic cells to the collection tube. The narrow magnetic gap between the two opposed horseshoe magnets provided a strong field in which to retain magnetic cells.

FIG. 4

Lungs from white New Zealand male rabbits (2-3 kg) were perfused with collagenase (50 mg%) and elastase (1.1 mg%) through the cannulated pulmonary artery. Cells were removed from the lungs either by mincing and shaking the lung to dislodge cells or by harvesting the cells directly through the trachea from intact lungs. Viable cells were counted with a standard hemocytometer in 0.1% trypan blue and identified with fluorescence microscopy, modified Papanicolaou staining and electron microscopy both before and after magnetic removal of macrophages and sizing of cells on nylon filters. n=4

FIG. 5

5a. Type II cells were obtained by methods described in this report. 10 X 10^6 cells were placed in a 1.8 ml Clark type O₂ electrode with glucose free HEPES buffer and 200 ul fetal calf serum. After a 5 minute equilibrium period O₂ consumption was measured during 10 minute periods. During one of these measurements the total chamber concentration of Na succinate was brought to 10 mM and O₂ consumption measured. 5b. Values for O₂ consumption also obtained from Fe₃O₄ containing macrophages which were taken from the separation chamber after type II cell enrichment. Values were expressed as n moles O₂ consumed/ 10^6 cells/hr. n=20.

FIG. 6

6a. Type II cells were isolated by methods described in this report. 10 X 10^6 cells were incubated with either 4uCi ¹⁴C Na acetate (20 Ci/ Mole) 6a or 10.7 uCi ¹⁴C Choline Chloride (5.37uCi/uMole) 6b for 3 hours. The reaction was stopped by adding 20 volumes of Folch reagent and the lipids

separated according to methods described. Rates of incorporation were determined and expressed as nM/ 10^6 cells/hr. Rates were compared with non-paired student T. n=6.

FIG. 7a & b

7a shows a preparation of type II cells stained with a modified Papanicolaou stain. Cells appeared granular with darkly staining inclusion bodies thought to be the surfactant-containing lamellar bodies characteristic of type II cells. 7b is a mixed preparation of cells, containing many pulmonary alveolar macrophages. They appear larger and stain more uniformly blue. 250X

FIG. 8a & b

8a cells were isolated according to procedures described in the Materials and Methods section. For identification with fluorescence microscopy, cells were incubated for 5 min. at room temperature with lug/ml Acridine Orange and viewed through a Nikon fluorescence microscope with excitation energy at 466 nm and emission at 530 nm. Type II cells were identified by their intensely fluorescing appearance as compared to macrophages which displayed more granular diffusely fluorescing properties. A number of macrophages can be observed in this preparation. 8b. For comparison a phase contrast micrograph is shown in Fig. Both show the same field of cells. 250X

FIG. 9

Electron micrograph of isolated type II cell from rabbit lung. Inclusion bodies appear vacuolated but do contain various amounts of osmiophilic material. Membranes appear normal and the cytoplasm is stained uniformly.

FIG. 10

Electron micrograph of magnetite containing alveolar macrophage obtained from rabbit lung and retained in magnetic separation chamber. The granular appearance of the plasma membrane is due to non-specific binding of Fe_3O_4 particles. Round inclusion bodies are phagosome vacuoles of surfactant-like material which were cleared from the alveolar region by the macrophage.

FIG. 11

25×10^6 cells recovered from the isolation procedure described in this report were further separated on a Beckman JE-6 elutriator rotor and each fraction evaluated by electron microscopy, modified Papanicolaou stain~~s~~ and fluorescence microscopy as indicated in materials and methods. Recovery from elutriator head was 68%. Figures ^{above} about error bars indicates % Type II cells in each fraction. A = 2.

Table 1. Summary of Characteristics of Cells Isolated by Recirculatory Perfusion, Sizing and Magnetic Macrophage Removal

	Cells Harvested by Lavage		Cells Harvested by Mincing	
	Lung Type II Cell Preparation	Macrophage Preparation	Lung Type II Cell Preparation	Macrophage Preparation
Total Cells ¹ $\times 10^6$	20 \pm 7	8 \pm 2.1	180 \pm 40	36 \pm 8
Type II Cells 1 $\times 10^6$	11 \pm 3	7.6 \pm 1.4	37 \pm 9	32 \pm 7
% Type II Cells 1	55	95	21	88
% Viability 1	96	93	94	90
O ₂ Consumption nmoles ¹ O ₂ /10 ⁶ Cells/Hr		84 \pm 4	88 \pm 6	
14C Acetate Incorp. nmoles/10 ⁶ Cells/Hr			0.44 \pm 0.1	0.18 \pm 0.06
14C Choline Incorp. nmoles/10 ⁶ Cells Hr			0.115 \pm 0.01	0.030 \pm 0.005

n = 20

Table 2. Contaminants in Type II Cell Preparations

Relative Contribution of Non Type II Cells to the Total Contaminants (100%)					
	Macrophages	Ciliated Epithelial Cells*	Clara Cells	Lymphocytes	Eosinophils and Neutrophils
Harvested by Lavage	9 \pm 6	7 \pm 3	5 \pm 4	75 \pm 14	4 \pm 2
Harvested by Mincing	8 \pm 8	12 \pm 5	5 \pm 4	73 \pm 20	2 \pm 2

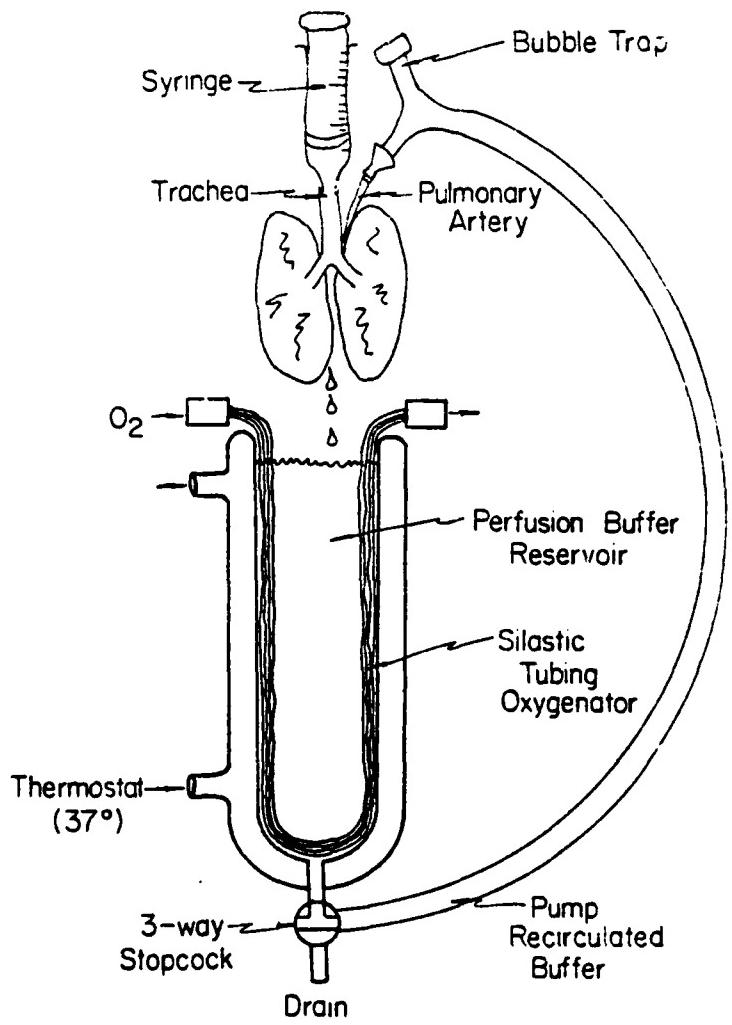
Cell types were identified by transmission electron microscopy. With each preparation, 100 contaminant cells were counted.

*Mann-Whitney U Test comparison between Methods 1 and 2 demonstrated significant differences in populations of non type II cells in ciliated epithelial cell contamination only. N = 20. *Signif. p 0.01.

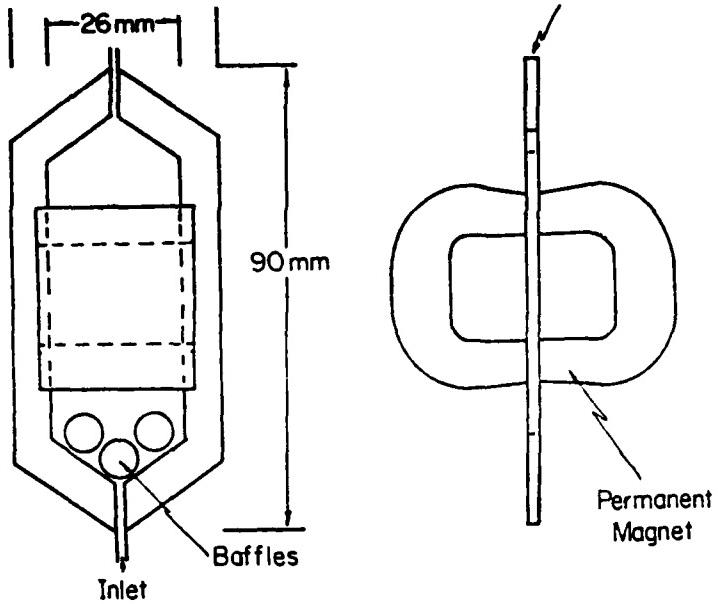
Acknowledgements

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Fig 1



Perfusion Apparatus



Separating Chamber

Fig 3

- A. Buffer Reservoir
- B. Pump - Flow Rate
0.884 ml/min
- C. Cell Suspension
- D. 3-way Stopcock
- E. Loading Chamber
- F. Silastic Tubing
- G. Separating
Chamber
- H. Funnel
- I. Collecting Tube

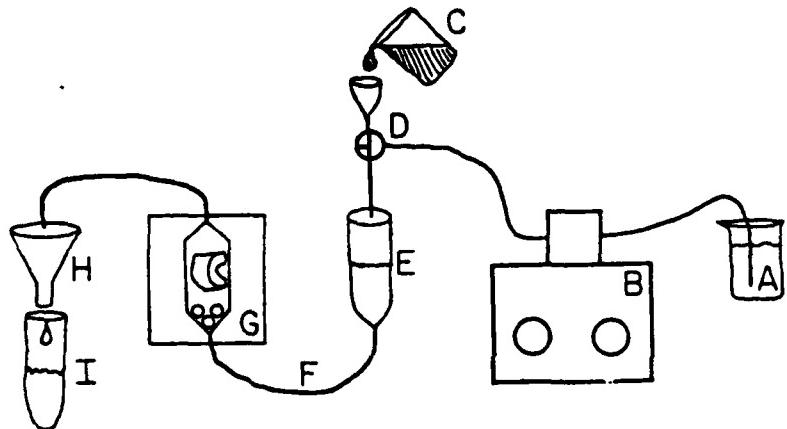


Fig 2

Cell Separator

Figure 4

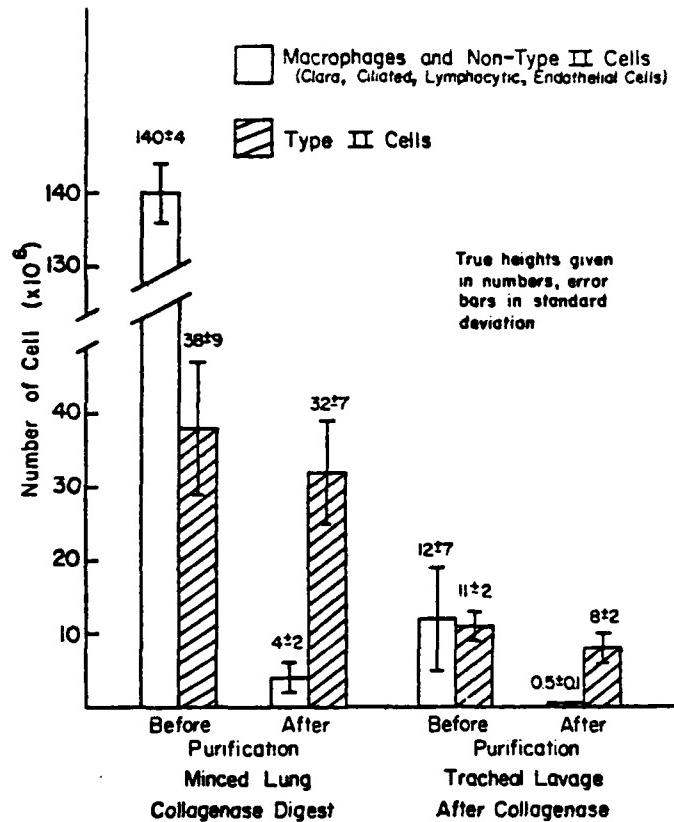


Fig 4

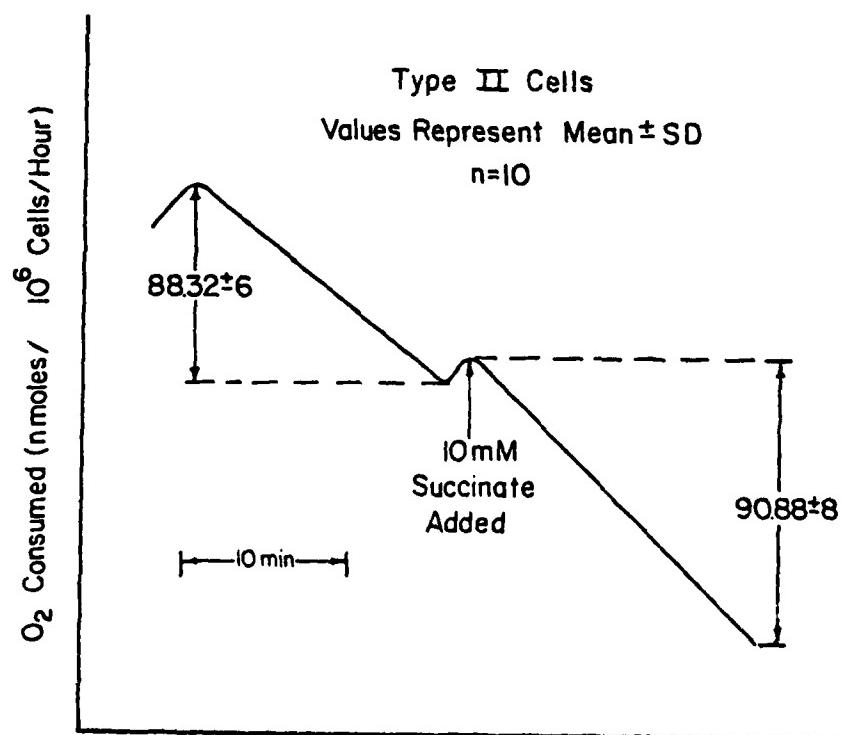
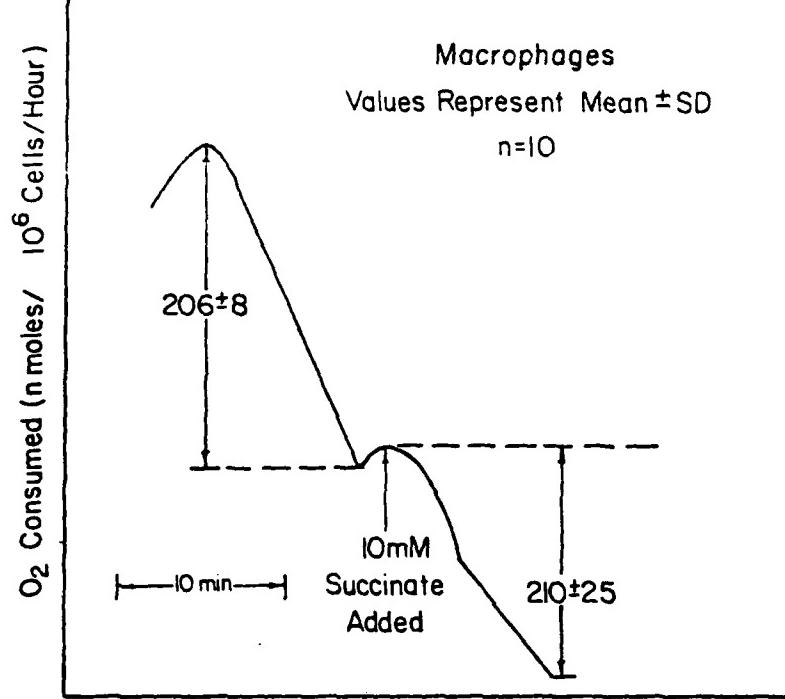


Fig 5

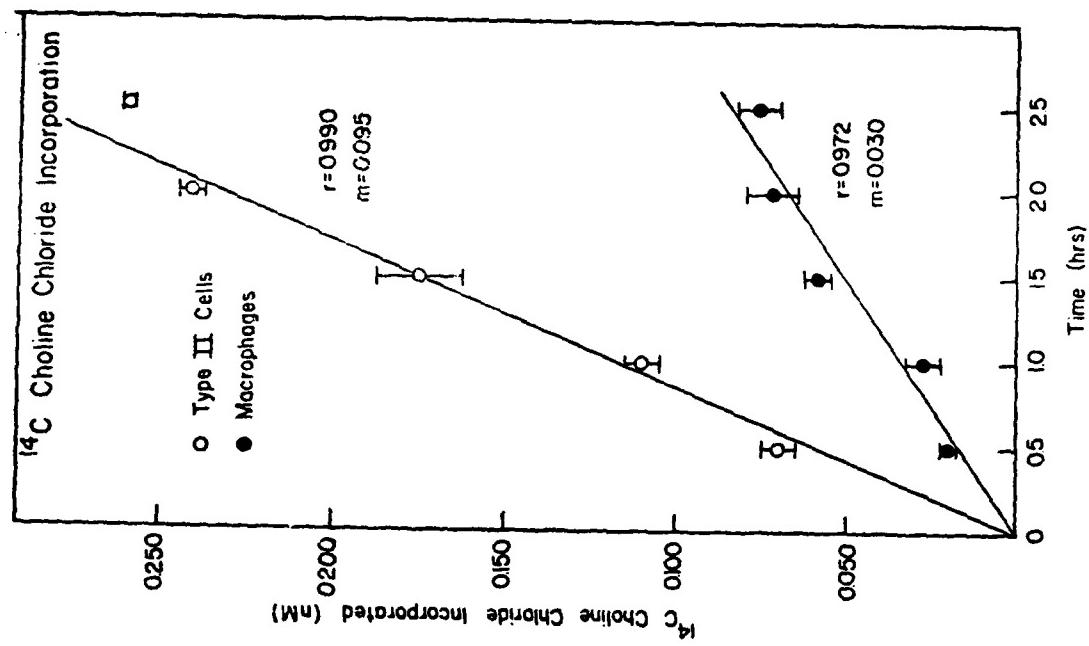
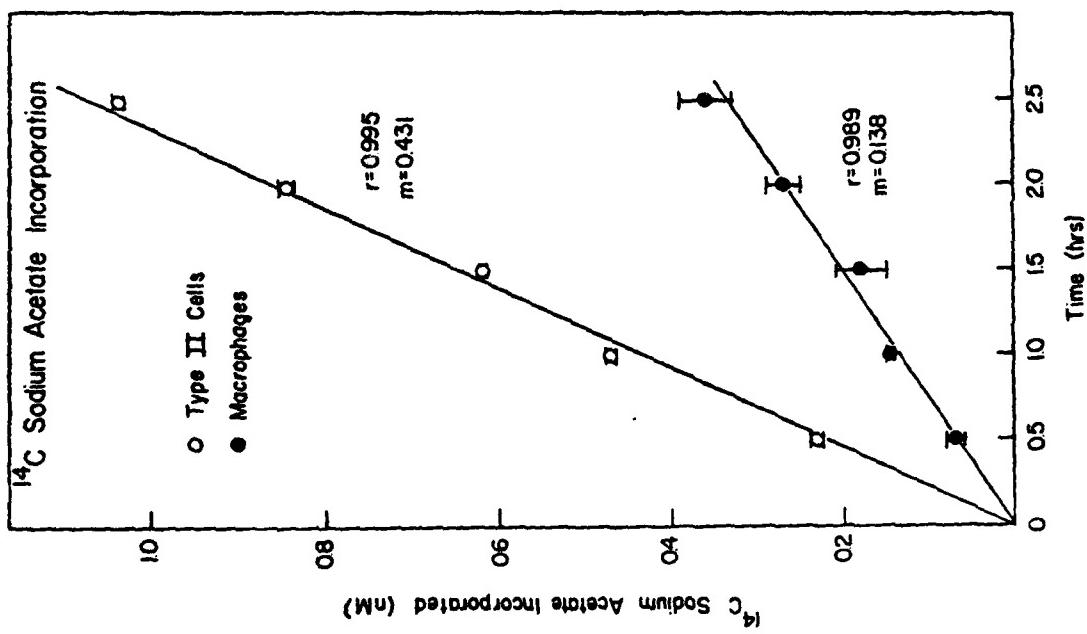
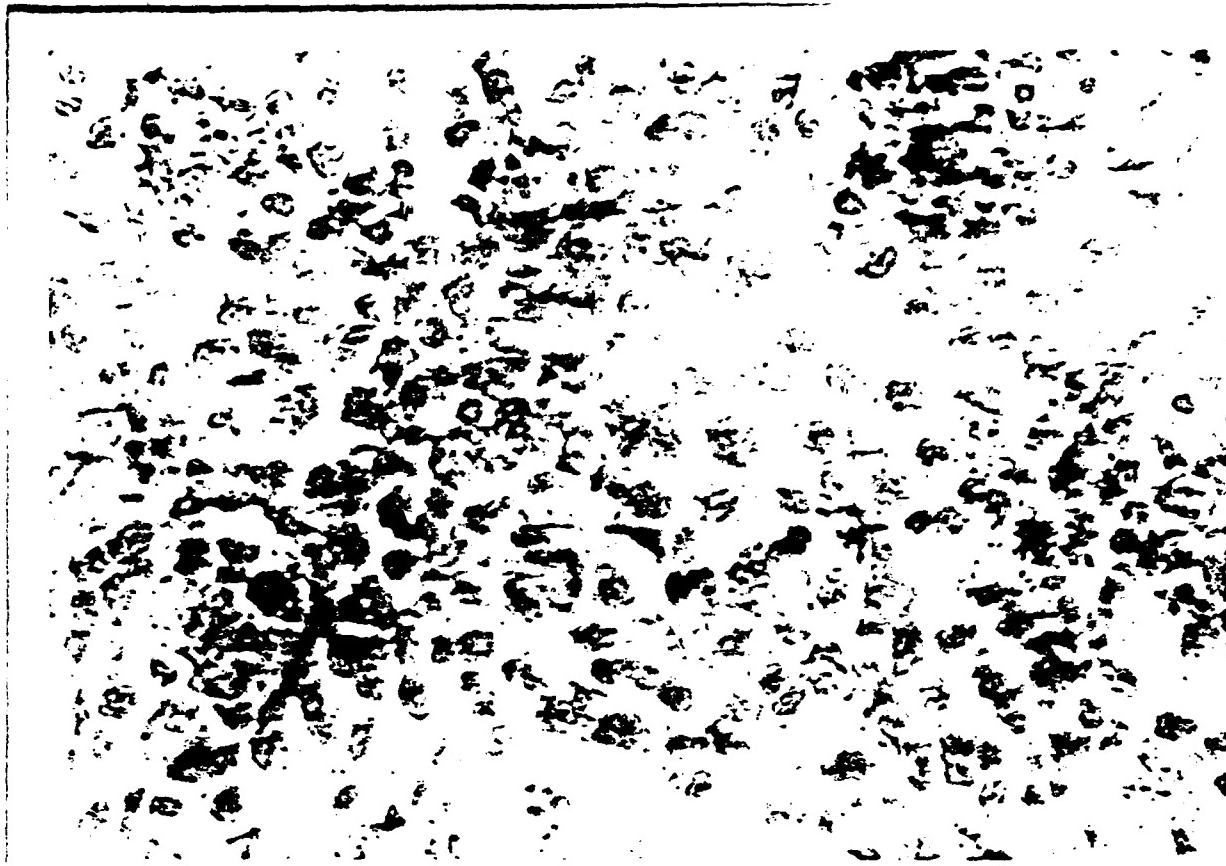


Fig. 6





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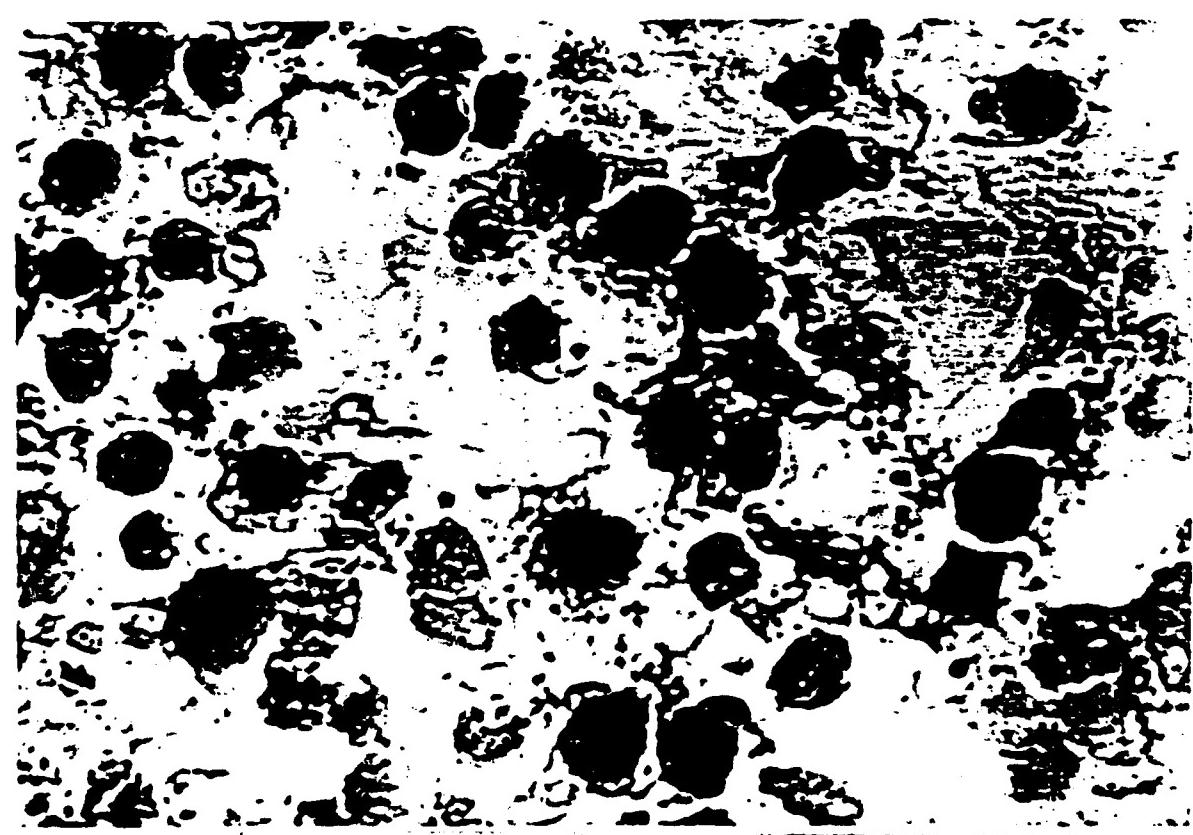


Fig 2

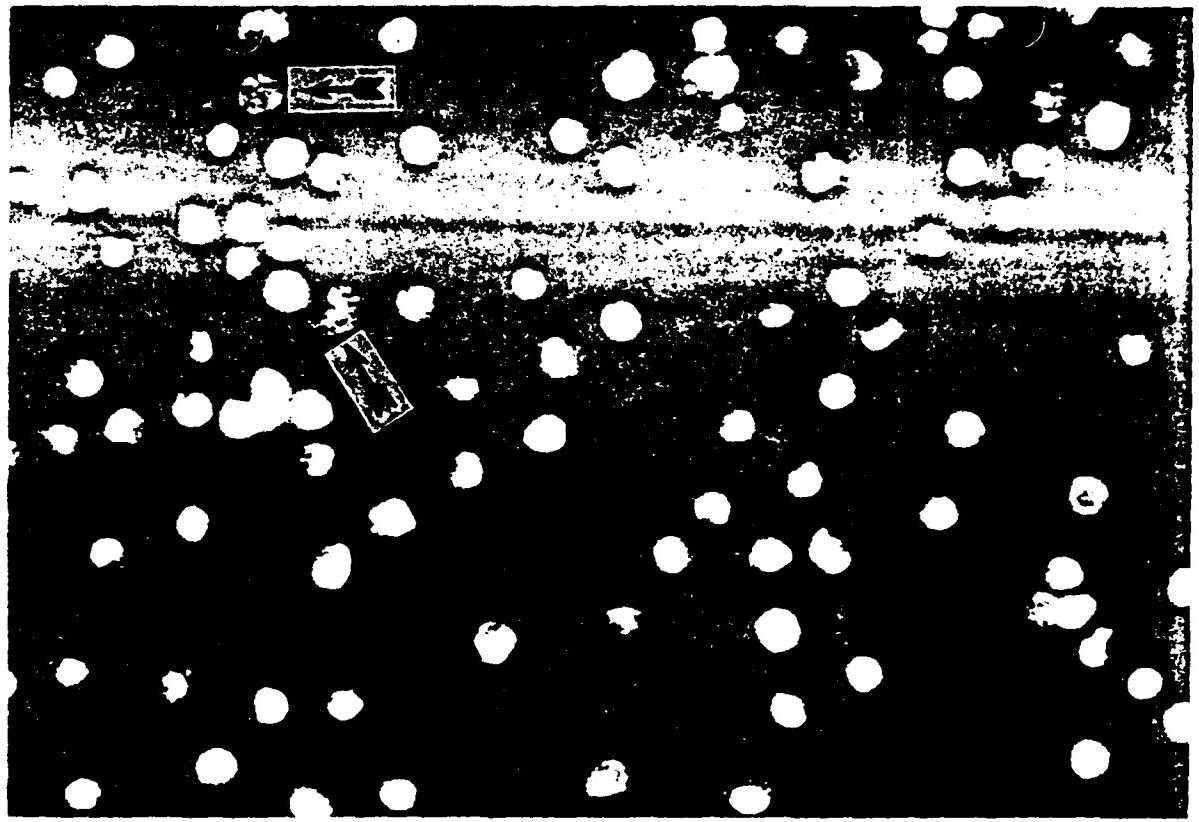
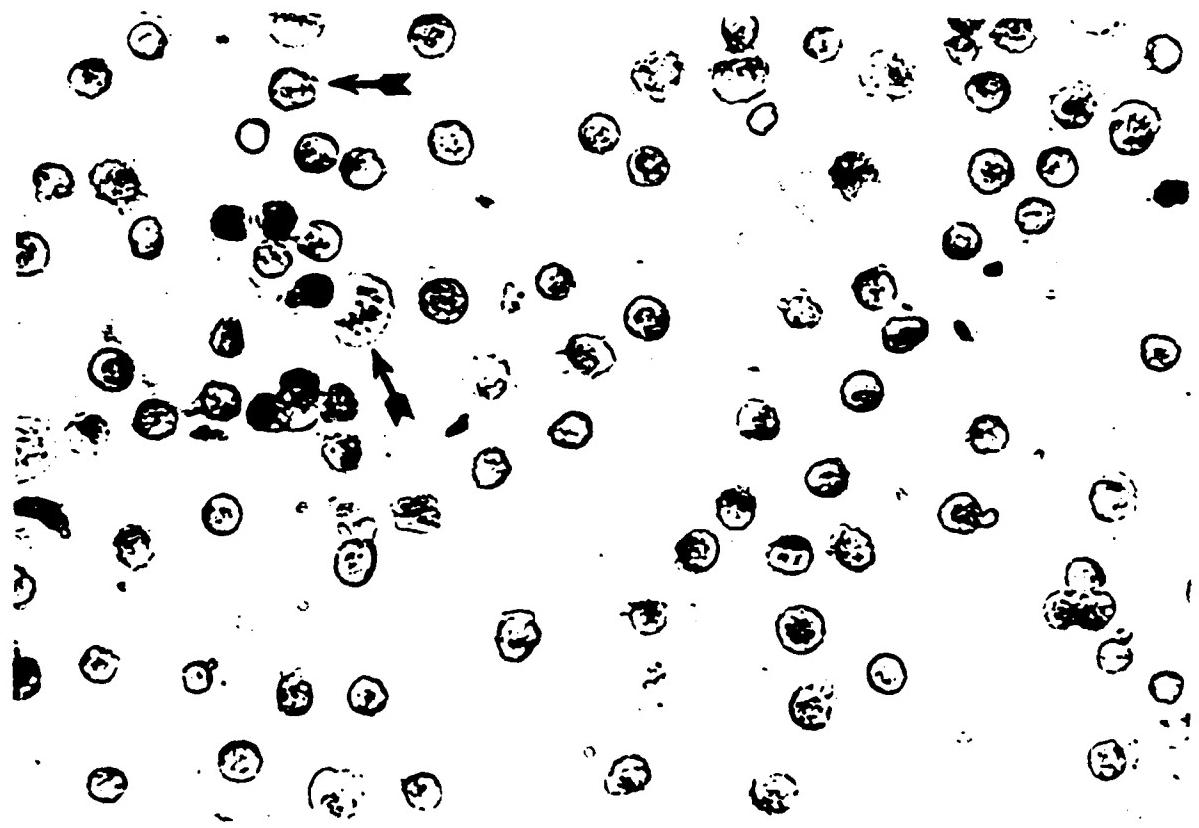


Fig 4



Fig 10



ELUTRIATION SEPARATION OF TYPE II CELLS

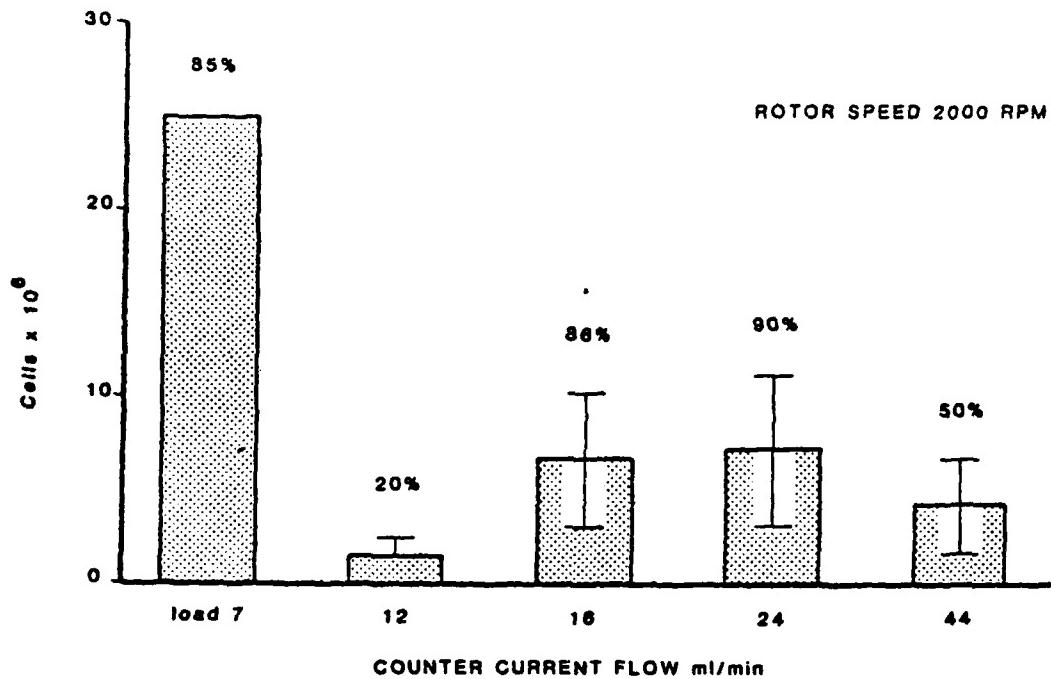


Fig 11

Isolation and Metabolic Characteristics of Lung-Cell Types

M. Lafranconi, K. Brendel, and I.G. Sipes

**Department of Pharmacology
College of Medicine
University of Arizona
Tucson, Arizona 85724**

Running Title: Isolated Lung Cells

**Corresponding Author: Mark LaFranconi
Department of Pharmacology
Arizona Health Sciences Center
Tucson, Arizona 85724
(602) 626-7843**

Abstract

A number of pulmonary toxins have a surprisingly specific site of action within the lung. Silica particles cause lysis of pulmonary alveolar macrophages, paraquat administration results in lipid peroxidation and type I cell death, urethan can cause adenomas of type II cell origin, pyrrolizidine alkaloids specifically damage pulmonary endothelium while 4-ipomeanol or carbon tetrachloride results in focal necrotic lesion of clara cells. Studying these events with isolated cell types would provide good specificity of response without interference from other cell types. Unfortunately few cell types in the lung have been successfully isolated. Methods available to isolate pneumonocytes generally take advantage of one or a combination of 4 basic differences in cells. 1. Availability 2. Density 3. Size 4. Specific cellular functions Macrophages have been isolated based on availability by lavage. Cell dispersal techniques become important in cell isolation procedures relying on density, size or specific cellular function differences. Because of the extensive connective tissue matrix in the lung, obtaining cells in a monodispersed form for separation has proven to be difficult. Density differences between pneumonocytes is slight but has been used to isolate type II cells. Size differences either through unit gravity velocity sedimentation or elutriation techniques have been used to isolate type I and II cells as well as partial enrichment of clara cells and macrophages. Specific cellular functions such as differential adherence or culture techniques or surface charge differences have been used to prepare cell fraction rich in macrophages, type II cells, endothelial cells and clara cells. Combinations of techniques have been used successfully but all are time consuming, requiring up to 8 hours. A technique using

critical enzyme placement, magnetic removal of macrophages and sizing of cells on nylon mesh screens results in a preparation of type II cells of over 85% purity. The prime advantage of the method is the short preparation time of less than 2 hours.

Isolating specific cell types is a promising area of lung toxicology. For some time now evidence has been building to suggest that cells within the lung may respond uniquely to noxious stimuli. Silica particles of less than 5 microns administered intratracheally to rodents will cause lysis specifically of pulmonary alveolar macrophages (3,10,45). When urethane is administered to mice IP within a matter of weeks proliferation of Type II cells ensues leading to the formation of adenomas in the lung of primarily Type II cell origin (53). Paraquat in rats can cause free radical formation and lipid peroxidation in Type I cells causing destruction of these cells (52). Endothelial cells are the first cells of the lung to encounter systemically applied agents and so are readily damaged. But a pyrrolizidine alkaloid called monocrotaline when administered in low doses for extended periods of time can alter uptake of serotonin by these cells while enhancing the activity of angiotensin converting enzyme (30). Carbon tetrachloride or 4-Ipomeanol administered parentally will cause focal lesions within the clara cells of the lung (6,7). Isolated cell types from the lung would provide the opportunity to study toxic responses from the cell specific agents without possible biochemical contribution from other cell types thereby allowing good quantitation. In addition, normal metabolism and physiology could be studied using isolated cells and these agents as possible chemical probes.

While there are over 40 cell types within the lung (54) only a few have been successfully isolated. The impetus for most of these isolations was the desire to study normal morphology and physiology. For example, pulmonary alveolar macrophages were initially isolated to study the mechanisms of phagocytosis (44). Type II cells were isolated to study surfactant synthesis (34). Only recently have efforts been made to isolate cell types from the lung to specifically study toxic responses (12,13).

The purpose of paper then, is to briefly outline the methods currently available for isolating cells from the lung and to discuss some of the problems associated with each.

Methods developed to isolate cells as they were required by investigators. Naturally, the simplest methods were exploited first, followed by methods of increased complexity. Despite the various methods available in the literature to prepare enriched fractions of lung cells they all rely on one or a combination of 4 basic properties to separate cells. These are differences in: 1) Availability; 2) Density; 3) Size; 4) Specific cellular functions. This last is a catch all category we will discuss later.

Availability

Isolating cells on the basis of availability is the simplest method to date and is one of the few success in cell isolation attempts from the lung. Pulmonary alveolar macrophages (PAMS) are free roaming cells in the lung alveolar areas. Thirty - 100×10^6 cells can be removed from a single rabbit lung by simple lavage (44) (See Fig. 1). Cells obtained in this way are greater than 90% macrophages with a viability greater than 90%. Further enrichment can be achieved by culture techniques (11). When cells are plated in tissue culture medium such as minimal essential medium supplemented with 10% fetal calf serum macrophages will adhere within 2 hours. The non adherent cells include non-viable macrophages, lymphocytes, red blood cells, some Type II cells and others.

Cells isolated in this way have been used, in studies investigating mechanisms of phagocytosis (44). However, other experimenters have used these

cells to study the toxicity of a variety of airborne contaminants because the cells were easily obtained and because, presumably, the pulmonary alveolar macrophages were representative of all cells in the alveolus to airborne exposure (1,9,32). Isolated pulmonary alveolar macrophages were used to show toxic effects of silica particles. These cells showed extensive *in vitro* degredation and lysis within 8 hours of application of silica particles of less than 5 microns (3,4,6). Using a similar system other groups were able to show elevated collagen synthesis in cultured fibroblasts when incubated in medium obtained from silicotic macrophages (2,8). In other experiments Cd⁺⁺, Hg⁺⁺, Pb⁺⁺ and Ni⁺⁺ inhibited release of reactive oxygen species during phagocytosis as well as decreasing oxygen consumption and glucose metabolism both at rest and during phagocytosis in isolated pulmonary alveolar macrophages (9).

Cell Removal

Unfortunately, the remainder of the cells in the lung are not as easily obtained. The cells of the lung are, for the most part, immobilized through a variety of attachments - the most important being connections to an extensive collagen containing matrix (25,28). Removing the cells from this matrix is the most recalcitrant problem in isolating cells from the lung. For a comprehensive review of this subject see Gould (25). To conduct any of the remaining procedures for isolating cells, they must be put into a monodispersed form - individual cells without attached basement membranes or clumping to form cell to cell attractions and all this while maintaining the cells in a viable state.

There are 3 primary methods available to disperse cells: mechanical, chemical or enzymatic means (25). Mechanical procedures provides a physical

disruption and shearing process which interrupts the cell-matrix bonds as well as cell to cell connections but unfortunately these methods are damaging to the cells and results in poor yields when used alone. Chemical means have not been used exclusively in removing cells from the lung either. The concept used in these procedures is to chemically chelate divalent cations such as Ca^{++} or Mg^{++} with EDTA or EGTA, thereby disrupting the Ca^{++} dependent desmosomes (25,58). Enzymatic means have been used with some success although cellular damage does occur as suggested by changes in surface receptors (15). Intercellular alterations have also been reported. Proteases caused a 4 fold reduction in NADPH cytochrome C reductase activity in rat lungs (19).

Cell isolation from lungs is dependent primarily on the success of obtaining individual dispersed cells. In other organs such as the liver, this step has been readily achieved using a combination of the above methods of cell removal. Proteases perfused through the portal vein, Ca^{++} chelation, mincing and pressing the liver through 160 μm nylon meshes can provide approximately 500 million cells from the liver of a 200 gram rat (29). In contrast the lungs from a 200 gram animal will yield less than 10 million cells (unpublished observation). The difficulty in obtaining cells from mammalian lungs lies in the structure of the organ.

From the liver section shown in Fig. 2 it can be seen that cells are arranged in sinusoids with little structural support from extracellular materials. The parenchyma are arranged through cell to cell junctions via desmosomes. In the lung however parenchyma are arranged about a collagen extracellular matrix as well as cell to cell junctions in zonas occludens (25,28) (see Fig. 3). In addition, there are large liquid gas interfaces from the alveolar aspect. Attempts to dissociate the lung then are hampered by

tight cellular junction which inhibit access of enzymes, the recalcitrant nature of the cell junction to basement membranes, and the high surface tensions at air - liquid interfaces. However, through extensive lavage, mechanical disruption by mincing, and enzymatic dissociation some success has been achieved in removing cells from the lung (12,13,15,21,22,24,25,26,34, 35,36,40,48).

Specific and non-specific enzymes in various combination or separately have been used to dissociate the lung. The non-specific enzymes such as trypsin or the pronases have good proteolytic activity and release cells from the lung effectively (12,34,40). In rabbit lungs, 0.1% pronase Type I can release up to 300 million cells from a single rabbit lung (12,13). Because of the non-specificity of action of both trypsin and 742 pronases certain cellular damage does occur and viability of the preparation falls with time (19,22).

It therefore becomes a trade-off between viability and cell yield that investigators must deal with when trying to remove cells from the lung. For example, rat lungs treated with thermolysin, one of the commercially available pronases will yield 10 million cells from a single lung within 25 minutes, but viability begins to fall after 10 minutes (see Fig. 4).

Specific enzymes such as collagenase, elastase or others have the advantage of being less damaging to the cells but the effectiveness of the agents in freeing cells from the lung is diminished (15,25). In our lab less than 100×10^6 cells can be obtained from rabbit lungs when collagenase Type II is used. This is compared to over 300 million obtained with pronase (12,13).

Removing cells from the lung with the combination of techniques described earlier leads to as yet another problem. The enzymatic action on cells by the

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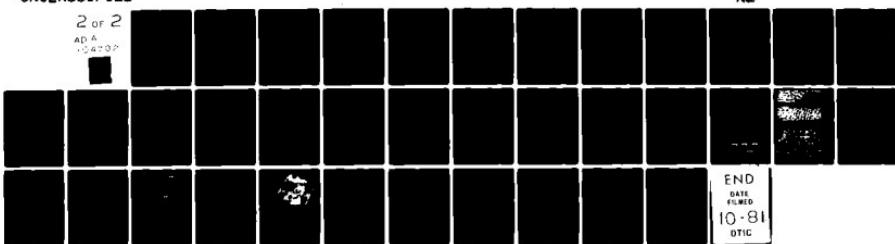
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proteases can cause altered surface proteins and change surface charges which contributes to cell aggregation. It is critical for successful cell isolation procedures that the cells are in a monodispersed form. Clumping can be avoided by adding protein to the medium such as bovine serum albumin up to 1% or fetal calf serum. Another technique used is to separate cells in the cold since clumping has been shown to be a function of temp (43). Divalent cations such as Ca^{++} or Mg^{++} will contribute to cell clumping so chelation of these materials is also used (25). Sometimes damaged cells will release DNA and the hgh viscocty of this material causes cell clumping which is prevented by adding DNAase to the medium, commonly in concentration of 0.05% (12,25). With these precautions monodispersed cells can be obtained from the lung and separation of cell types based on differences in density, size, or specific cellular functions can be achieved.

Density Separations

The first reported methods for isolation of Type II cells from the lung were based on density separations (21,34,40). With these method lungs were perfused to remove blood elements then lavaged to remove free macrophages. The lungs were then dissociated into monodispersed cells, primarily with trypsin, and the cells separated according to density differences on either albumin gradients (40), ficol gradients (21,34), or metrizamide (13).

The basis of this separation is derived from Stokes Law (42). The cells develop a velocity by centrifugal forces as a function of their radii. As long as the density of the cell is greater than density of the medium the cell continues to move. However, once the cell reaches a gradient with a density equal to or greater than that of the cell, the density function becomes 0 and the cell ceases to move.

With this method a number of investigators have enriched Type II cell fractions to greater than 50% (13,21,34,35,40). The process however is time consuming requiring 4-6 hours for completion. Furthermore, there is little within lung cells to provide much of a difference in density. Nucleic acids or protein concentrations may alter densities but levels of these components are not dramatically different between cell types in the lung. So therefore purification by density gradients has proven to be difficult. For instance Type II cells and smaller Pams layer on the same density gradient (34,40). A clever manipulation used by investigators to enhance macrophage and Type II cell separation is to provide a dense material such as BaSO₄ or fluorocarbon albumin mixtures which can be taken up into macrophages by phagocytosis, increasing their density enough to cause them to layer at a different zone than the Type II cells (21,34,40).

Using this method for isolating Type II cells and in conjunction with in vivo experiments mechanisms of surfactant synthesis and synthetic pathways with Type II cells have been investigated (For review see 4,35).

Size Separations

It has been proposed that size is the most accurate way of separating cells since lung cell sizes may vary from 10 microns for lymphocytes to 50 or 60 microns for Type I cells or macrophages (49). A number of methods have been published to separate lung cells based on size.

One of the procedures based on size is unit velocity sedimentation (31,48). In this instance velocity is developed as a function of the radius of the cell and the acceleration of gravity. The density term is always greater than 1. Monodispersed cells are obtained then placed in a chamber and allowed to separate according to velocities generated.

With this method of separation Type I cells of the lung have been prepared and enriched to nearly 70% homogeneity (48). This method, perhaps more than any other, is sensitive to cell clumping. It is also time consuming, requiring 6-8 hours of preparation to obtain cells in an enriched form.

This method has also been used to partially purify Type II cell fractions. After density separation of Type II cells the enriched fraction is then further subjected to unit gravity sedimentation which results in an enrichment of nearly 90% (40).

Another procedure to separate cells based on size is centrifugal elutriation. The same concepts apply here as for unit velocity sedimentation except the force for acceleration is centrifugal. Cells are introduced into the spinning rotor and pelleted to the outside of the chamber by centrifugal acceleration. By precisely increasing flow rates of the counter current buffer, cells are separated from the pellet according to their size.

Using this method fractions can be obtained which are enriched in pulmonary alveolar macrophages, Type II cells, clara cells, and others (12,13,26). It is faster than other methods mentioned so far for isolating cells, but when used alone only a limited enrichment is obtained - 60% Type II cells for an example (12).

Using a combination of centrifugal elutriation and density gradient centrifugation Type II cells have been enriched to nearly 90% purity (12,13,26). These methods of isolation have been used to study drug metabolic capabilities of cell types from the lung (12,13). They have also been used to study surfactant synthesis (24).

Specific Cellular Functions

As I mentioned earlier this last classification, specific cellular functions, is a mixed bag for separation techniques. It includes functions such as cell culturing, cloning, induction, differential adherence, surface charge differences, differences in membrane affinities, etc.

One of these methods, differential adherence has been used to isolate macrophages which we discussed already (11). But Type II cells have also been prepared from differential adherence procedures (15). With this procedure a preparation partially enriched in Type II cells, by some other means such as density gradient centrifugation, is plated in culture medium such as Dulbecco's modified eagle medium for 1 hour. At this point macrophages and some Type II cells adhere. The non-adherent cells are removed and plated again but for longer periods of time - 22 hours. In this second plating Type II cells adhere while non-adherent cells such as lymphocytes and red blood cells are removed with a fresh change of medium. With this method greater than 80% Type II cells can be obtained. Unfortunately, it is a time consuming process and in this preparation time and through the stresses of adapting to culture conditions cells might be altered physiologically since Type II cells reportedly rapidly dedifferentiate into some type of precursor cell (14,39). With this method however, it was demonstrated that adrenergic stimulation of Type II cells caused an enhanced release of surfactant synthesis (15).

A number of methods for culturing cells from the lung have been reported. Pulmonary alveolar macrophages have been maintained in cultures for weeks (11). Pulmonary endothelial cells harvested from lungs by retrograde perfusion of the pulmonary circulation with collagenase have been successfully cultured (27).

Limited success has been achieved with attempts to culture other cell types from the lung (14,16,17,18,23,51). Although a number of groups report successful cultures of Type II cells, recent reports have provided evidence that these cultured cells have altered phospholipid content and morphology compared to freshly isolated Type II cells or cells from the intact lung (41,50). None the less, cultured Type II cells have been used to examine surfactant synthesis (16) and recent reports demonstrate the existence of Aryl hydrocarbon hydroxylase activity in Type II cells in culture (55,56).

An innovative method has recently been developed to isolate clara cells based in part on size by elutriation separation then further separated on the basis of surface charge (13). When clara cell rich fraction are placed at the interface between a 2 phase aqueous solution with differnt charges the clara cells separate from a majority of the contaminating cells and appeared in the positively charged phase. Enrichments up to 70% were reported.

Another method which relies on a combination of techniques for isolating Type II cells has recently been developed by our lab (manuscript in preparation). This method employs critical enzyme placement, magnetic removal of macrophages and cell sizing to rapidly prepared Type II cells.

As with most of the procedures previously mentioned the initial step is to prepare a monodispersed suspension of pneumonocytes. To accomplish this cold isotonic saline is perfused through the cannulated pulmonary artery to remove free blood elements. Once the lungs have perfused white, they are attached to a continuous perfusion apparatus and perfused in a recirculatory manner with a HEPES buffer containing 128 mM NaCl, 5 mM K₂SO₄, 5 mM Kcl, 2.5 mM Sodium Phosphate buffer pH 7.4, 17 mM HEPES (N-2-hydroxyethyl piperazine N-2-ethane sulfonic acid), 5.5 mM Dextrose and 10% V/V fetal calf serum (Fig. 9).

At this time the lungs are lavaged 3 times through the cannulated trachea to remove free macrophages. Subsequently 20 ml of colloidal magnetic iron oxide (Fe_3O_4) are instilled via the trachea. With this the lungs take on a black appearance. This provides for phagocytosis of magnetic Fe_3O_4 particles by remaining macrophages. After 20 minutes of incubation at $37^\circ C$ the lungs are again lavaged to remove excess iron oxide. The lungs are then treated with a mixture of enzymes to dissociate cells from the matrix. Collagenase Type II (Worthington Biochemical Corp) 50mg/100 ml and 1.1 mg/100 ml elastase (11 mg/ml Sigma) are added to the perfusion reservoir in fresh HEPES buffer without fetal calf serum and perfused through the pulmonary artery in a recirculatory manner. At the same time collagenase and elastase containing buffer is introduced to the alveolar areas through the trachea. In this way the enzymes necessary for dispersing type II cells are in contact with the cells from both the epithelial and endothelial aspects.

Following a 10 min incubation at $37^\circ C$ freed cells are removed from the lung by one of two methods. In one procedure cells are recovered from the lung by lavage with fresh buffer. This results in a small yield of cells per lung - often less than 20×10^6 , but the initial purity is almost 60% type II cells. When more cells are required another method of harvesting cells developed by other workers (40) is used. The lungs are removed from the perfusion apparatus, minced then shaken in fresh buffer supplemented with 10% fetal calf serum for 10 min. Cells are then separated from the tissue blocks by filtering the mixture through a 200 μm nylon mesh screen. With this procedure approximately 180×10^6 cells are obtained from a single rabbit lung but with only 21% type II cells.

Irregardless of the method used to harvest cells from the lung, the preparations are then partially enriched by consecutive sizing on nylon mesh

screens of 160, 40, and 20 um. The 160 um grid removes cell clumps and broken pieces of matrix that may be present. The 40 um and 20 um sieves retain large macrophages, type I cells as well as some ciliated and non ciliated bronchial epithelial cells and endothelial cells.

At this point the cell preparation contains mostly type II cells, small macrophages and lymphocytes. The macrophages are then removed by passing the preparation through a magnetic separation chamber. The cells are slowly pumped past a narrow gap contained in a strong magnetic field. Macrophages containing magnetic iron oxide are retained in the chamber while non-retained cells, including type II pneumonocytes are collected.

With this system cells obtained by lavage can be enriched to greater than 90% while cells obtained through minced lung preparations can be enriched to greater than 85% type II cells. The prime advantage of the minced lung preparation is the increased number of type II cells obtained, 32×10^6 from a single rabbit lung compared to 8×10^6 from lavage. The unique feature of this preparation is the time savings. Commonly type II cell isolations require 4-8 hours for completion while this method can be used to prepare cells in less than 2 hours.

Identifying cells obtained from various isolation procedures is an important but difficult task. Because of the array of cell types in the lung it becomes important to be able to identify which cells are present in the preparation. In situ cell identification is, in part, accomplished by landmarks and location or by immunohistochemical techniques. But with isolated cells there are no landmarks or location clues and the proteolytic activities of the isolation procedure may alter the antigenic characteristics of the cell. When cells have unique surface receptors these receptors can be

bound to an antigenic ligand then visualized with immunohistochemical techniques. Unfortunately few surface receptors are unique in the lung that can be exploited at this time. One, the angiotensin converting enzyme on the surface membranes of endothelial cells has been used to identify endothelial cell preparations (27).

Other techniques used to identify cells take advantage of specific biochemical anomalies the cell. Clara cells have been histochemically identified by nitro blue tetrazolium which is reduced in clara cells to a purple formazon (13). Type II cells have been identified by a number of techniques. When incubated with phosphine 3R or acridine organ for 5 minutes Type II cells with fluoresce intensely yellow green while macrophages fluoresce diffusely (38,40). Type II cells are also identified by a modified papanicolaou staining in which Type II cells show darkly staining inclusion bodies while other cell types stain more uniformly (34). Still another method is electron microscopy. Type II cells are identified in this way by lamellar inclusion bodies (34,40). However, the morphology of the lamellar bodies may be significantly different from species to species (33). The micrograph in Fig. 4 is a Type II cell obtained from a rabbit, while Type II cells from rats have a more layered appearance.

Still another means of identifying cells is based on their separation properties observed in other methods of isolation. For example, Type II cells isolated by our lab with an apparent purity of 85% were further separated by centrifugal elutriation. The pattern of distribution of cells obtained at various flow rates corresponds to distributions of Type II cells reported by other groups with the majority of the cells recovered collected from fractions separated with 16 and 24 ml/min counter current flow rates (12,13,26).

Viability of cells obtained have been determined by trypan blue exclusion or other vital dyes such as erythrosin B (47) but other indexes of viability have been used also. Oxygen consumption or glucose utilization are both sensitive indices of viability (20,57). Here in Fig. 5 is a trace obtained from Type II cells of the rate of oxygen consumption expressed as nmoles/million cells/hour. The rate is linear over the ten minute time course. In addition, there was no significant changes in rate of utilization after addition of 10 mM sodium succinate, a mitochondrial substrate. This procedure is used to demonstrate intact plasma membranes since sodium succinate will normally not gain access to mito-chondria unless plasma membranes are damaged (5). The second trace is obtained from the macrophages which were collected in the chamber. Note that these cells have a much greater rate of oxygen consumption than do Type II cells.

Another index of viability is measurement of synthesis functions. Type II cells, because of their role in surfactant synthesis, contain high levels of disaturated phosphatidyl choline, a phospholipid relatively uncommon in other cell types (35,37,39). By supplying radio labeled precursors for phospholipid synthesis it is possible to monitor rates of synthesis of phosphatidyl choline (35,40). In Fig. 6 incorporation rates are linear for over 2 hours. This same experiment can also be used as a crude means of identifying the cell preparation. Type II cells have elevated rates of incorporation of radiolabeled precursors into disaturated phosphatidyl choline compared to other cell within the lung. Note the low rate of synthesis in macrophages. Thus, with a high rate of synthesis there is added support that the cells are sufficiently enriched in Type II cells.

Conclusions

There are a wide array of techniques used to isolate cells from the lung. Most methods rely on obtaining viable monodispersed cells from the lung matrix then separating these cells into various fractions either based on differences in density, size or some particular cellular function. Most of the successful procedures employed have been obtained through a combination of these techniques.

Perhaps with the existing techniques other cell types in the lung could be purified as needed for investigations. With isolated cell type and specific chemical or biologic probes normal physiology and biochemistry could be studies in depth. In addition, mechanisms of action of toxins, drugs, or pathogens could be further investigated.

Future techniques for isolating cell types may rely on electrophoretic separations, affinity chromatography, flow microfluorimetry or improved culture techniques. Objectives of new techniques would be improved yield, viability, purity and reproducability along with perhaps a shorter preparation time.

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LEGENDS TO FIGURES

Fig. 1

Example of preparation of cells based in part on availability - a modification of the method of Myrvik et al (44). Lungs are lavaged with tissue culture medium, centrifuged at 300 x g for 5 min then washed with fresh medium which results in a preparation of pulmonary alveolar macrophages of greater than 80% purity. Further enrichment is achieved by plating cells under tissue culture conditions. Adherent cells are greater than 99% pulmonary alveolar macrophages (11).

Fig. 2

200 x H & E stained liver section. Cells are arranged in sinusoidal pattern with cell to cell junctions occurring via desmosomes. Little or no connective tissue is apparent. Using 50 mg type II collagenase/100 ml buffer, mincing, sizing and EDTA chelation of Ca^{++} and Mg^{++} 500×10^6 hepatocytes can be obtained from a 200 g rat (29).

Fig. 3

200 x H & E stained lung section showing alveolar areas. Cells are arranged in tight zones occludens on an extensive collagen containing basement membrane. There are also large areas of air-liquid interfaces which also hinder attempts to remove cells. Methods available to remove cells from this structure have used extensive lavage, protease treatment and mincing. With powerful proteases such as trypsin (up to 1% w/v) or thermolysin (up to 20 ug/ml) only 10×10^6 cells could be obtained from a 200 g rat.

Fig. 4

Male Sprague-Dawley rats weighing 200 g were anesthetized and the pulmonary artery cannulated as well as the trachea. Lungs were lavaged 3 times with cold 0.9% saline and perfused through the pulmonary artery cannula to remove blood elements. Lungs were removed en bloc and perfused with buffer containing 20 ug/ml thermolysin (Sigma) for times indicated. Cells were counted on a standard hemocytometer and viability checked by trypan blue exclusion. (n=3 for each time point).

Fig. 5

Differences in cell densities have been used to separate pneumonocytes into fractions rich in alveolar type II cells. Monodispersed cells are placed upon gradients of either ficol, albumin or metrizamide and centrifuged. Cells move through the gradients with a velocity proportional to the square of their radii until they reach a density that is greater than or equal to their own at which point V falls to 0. Cells are then recovered at the appropriate density layer and washed. With this method type II cells have been prepared with greater than 50% purity.

Fig. 6

Separation by size via unit gravity velocity sedimentation has been used to separate type I cells from lungs of rabbits (48). Lung cells are prepared in a monodispersed form then placed on a shallow gradient (3% to 6% ficol in MEM) and allowed to settle through the system with velocities proportional to the square of their radii under 1 G acceleration. Cells are then removed in discrete layers by displacement with 10% sucrose.

Fig. 7

Elutriation methods have also been used to partially enrich fractions in type II cells (12,13,26) and clara cells (13) based on differences in their size. Cells are introduced to a spinning elutriator rotor and are pelleted to the outside of the chamber via centrifugal acceleration. A counter current of buffer which opposes the centrifugal forces can be increased in discrete units until forces generated by angular velocity and radii of the cells are less than forces resulting from counter current flow, at which point cells begin to migrate with the counter current buffer out of the system according to size.

Fig. 8

One specific cellular function that has been used to separate pneumonocytes is differential adherence to culture vessels. Type II cells have been prepared in this way (15). Cells previously partially enriched in type II cells by density separations are plated under tissue culture conditions. After 1 h non-adherent cells, primarily type II cells are plated into fresh vessels for 22 h. During this time type II cells adhere while non-adherent cells such as lymphocytes, RBCs and non-viable cells are removed with a change of medium.

Fig. 9

Lungs were removed from anesthetized animals and attached to the recirculatory perfusion apparatus. Warmed and oxygenated buffer was continuously perfused through the isolated organ. Free macrophages were removed by lavage through the cannulated trachea. Colloidal Fe_3O_4 was introduced to the alveolar regions via the cannulated trachea.

Fig. 10

After enzymatic dissociation with collagenase and elastase cells are harvested either by simple lavage in which case cell yield is less than 12×10^6 cells per rabbit lung, or alternatively by mincing lungs, shaking minced preparation in a 37 C water bath for 10 min to free dissociated cells in which case 140×10^6 cells can be obtained. In either case cells are then sized on nylon mesh screens of 160, 40 and 20 um opening size.

Fig. 11

To separate cells the loading chamber and separating chamber were filled with buffer and air bubbles removed from the lines and chambers. Monodispersed cells were introduced into the loading chamber and the stopcock rotated to allow the pump to move fresh buffer through the loading chamber, carrying cells through the separating chamber and into the collecting tube.

Fig. 12

Enlarged figure of separating chamber: Monodispersed cells were introduced to the chamber and past baffles which reduced turbulent flow. Cells moved through the magnetic field with a linear velocity of 2 mm/sec which allowed retention of Fe_3O_4 containing cells while moving non-magnetic cells to the collection tube. The narrow magnetic gap between the two opposed horseshoe magnets provided a strong field in which to retain magnetic cells.

Fig. 13.

Lungs from white New Zealand male rabbits (2-3 kg) were perfused with collagenase (50 mg%) and elastase (1.1 mg%) through the cannulated pulmonary artery. Cells were removed from the lungs either by mincing and shaking the lung to dislodge cells or by harvesting the cells directly through the trachea from intact lungs. Viable cells were counted with a standard hemocytometer in 0.1% trypan blue and identified with fluorescence microscopy, modified Papanicolaou staining and electron microscopy both before and after magnetic removal of macrophages and sizing of cells on nylon filters. n=4

Fig. 14

Electron micrograph of isolated type II cell from rabbit lung. Inclusion bodies appear vacuolated but do contain various amounts of osmiophilic material. Membranes appear normal and the cytoplasm is stained uniformly.

Fig. 15

25×10^6 cells recovered from the isolation procedure described in this report were further separated on a Beckman JE-6 elutriator rotor and each fraction evaluated by electron microscopy, modified Papanicolaou staining and fluorescence microscopy as indicated in materials and methods. Recovery from elutriator head was 68%. Figures ^{above} about error bars indicates % Type II cells in each fraction. n = 2.

Fig. 16

16^a(a) Type II cells were obtained by methods described in this report. 10 X 10^6 cells were placed in a 1.8 ml Clark type O₂ electrode with glucose free HEPES buffer and 200 ul fetal calf serum. After a 5 minute equilibrium period O₂ consumption was measured during 10 minute periods. During one of these measurements the total chamber concentration of Na succinate was brought to 10 mM and O₂ consumption measured. 16(b) Values for O₂ consumption also obtained from Fe₃O₄ containing macrophages which were taken from the separation chamber after type II cell enrichment. Values were expressed as n moles O₂ consumed/ 10^6 cells/hr. n=20.

Fig. 17

Type II cells were isolated by methods described in this report. 10 X 10^6 cells were incubated with either 4uCi ¹⁴C Na acetate (20 Ci/Mole) 6a or 10.7 uCi ¹⁴C Choline Chloride (5.37uCi/uMole) 6b for 3 hours. The reaction was stopped by adding 20 volumes of Folch reagent and the lipids separated. Rates of incorporation were determined and expressed as nMole/ 10^6 cells/h. Rates were compared with non-paired student T. n=6.

Table I. Contaminants in Type II Cell Preparations

	Relative Contribution of Non Type II Cells to the Total Contaminants (100%)				
	Macrophages	Ciliated Epithelial Cells*	Clara Cells	Lymphocytes	Eosinophils and Neutrophils
Harvested by Lavage	9 \pm 6	7 \pm 3	5 \pm 4	75 \pm 14	4 \pm 2
Harvested by Mincing	8 \pm 8	12 \pm 5	5 \pm 4	73 \pm 20	2 \pm 2

Cell types were identified by transmission electron microscopy. With each preparation, 100 contaminant cells were counted.

*Mann-Whitney U Test comparison between Methods 1 and 2 demonstrated significant differences in populations of non type II cells in ciliated epithelial cell contamination only. N = 20. *Signif. p 0.01.

Acknowledgements

This work was supported in part by a grant from the Office of Naval Research N00014-77-C-0506 and an NMIH training grant fellowship 1-I-32-ES0-7091 to WML. We gratefully acknowledge Dr. Eugene Gerner and Mr. David Holmes of the Department of Radiation Biology University of Arizona for their generosity in helping to conduct the elutriation experiments.

Fig. 1

PULMONARY ALVEOLAR MACROPHAGE PREPARATION

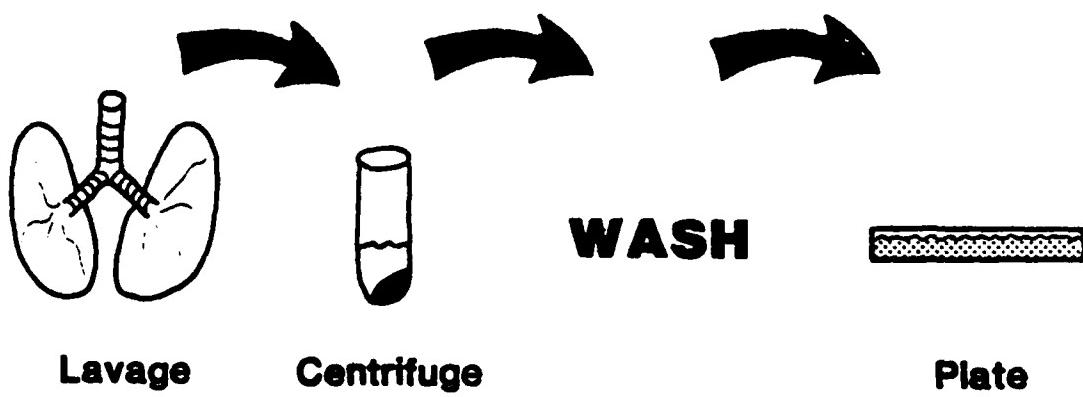




Fig 2

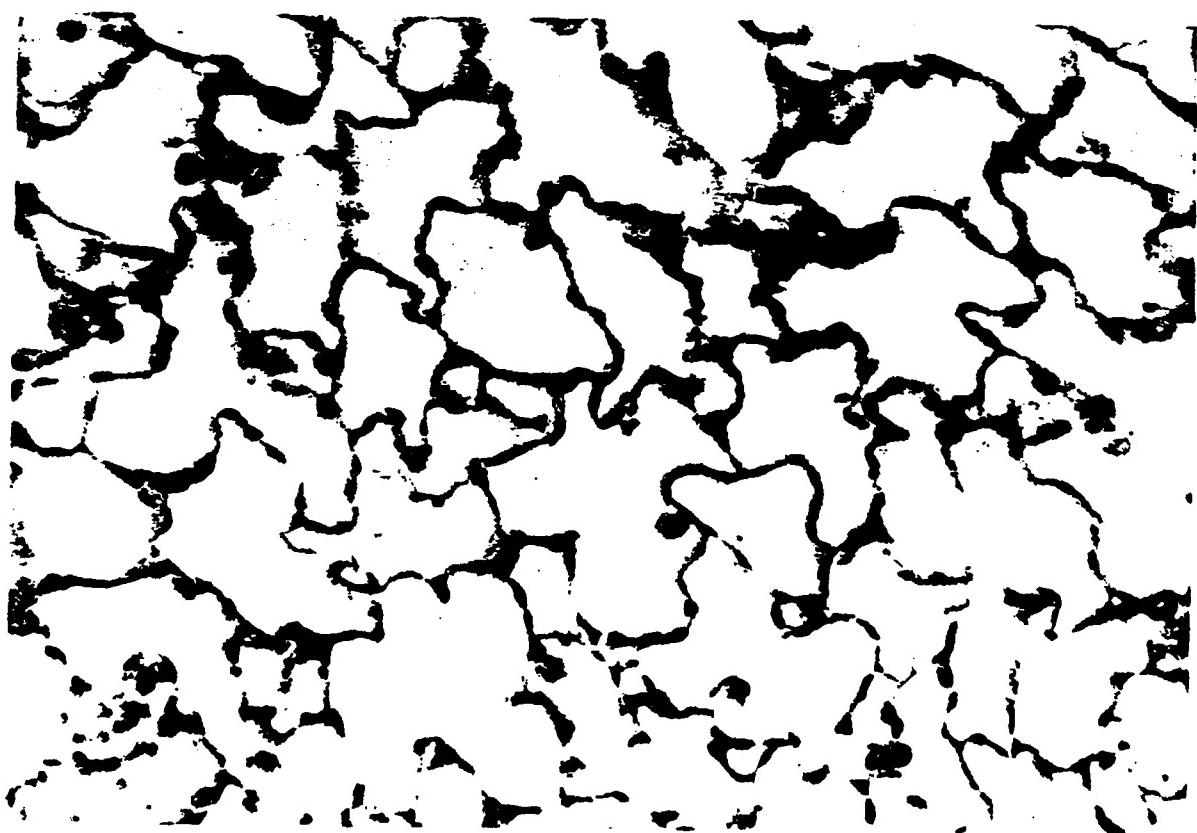
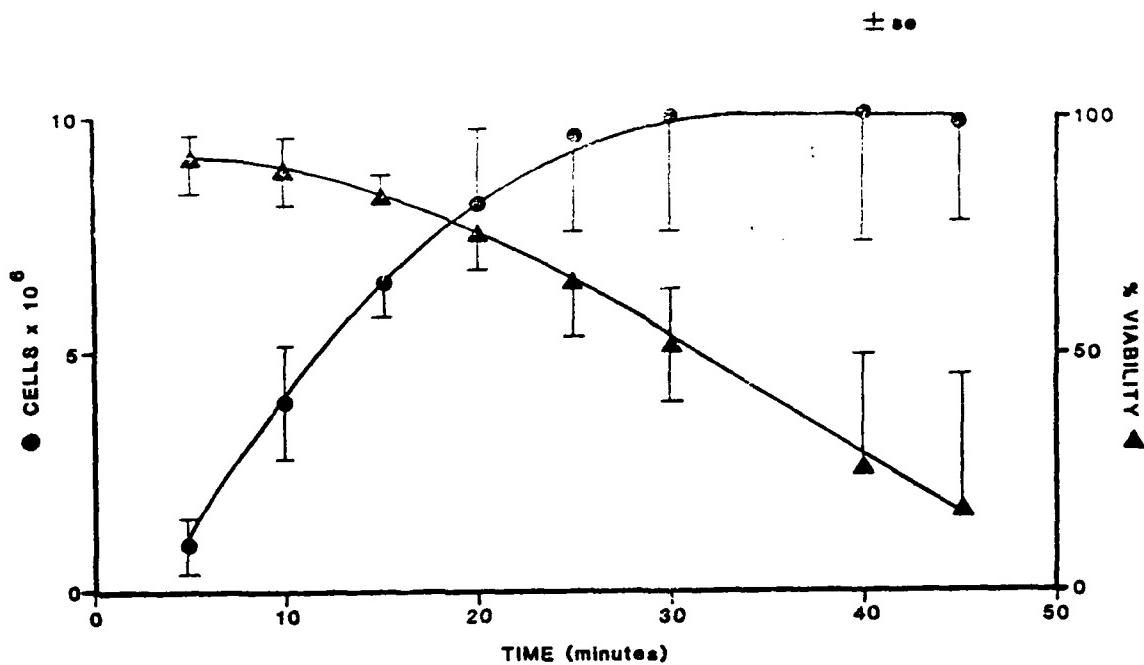


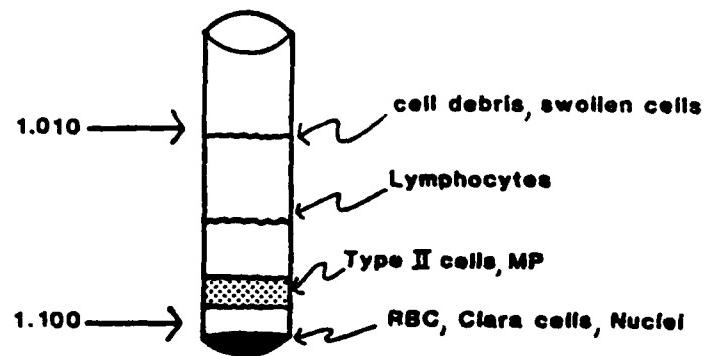
Fig 3

RAT LUNG THERMOLYSINE DISSOCIATION



SEPARATION BY DENSITY GRADIENT CENTRIFUGATION

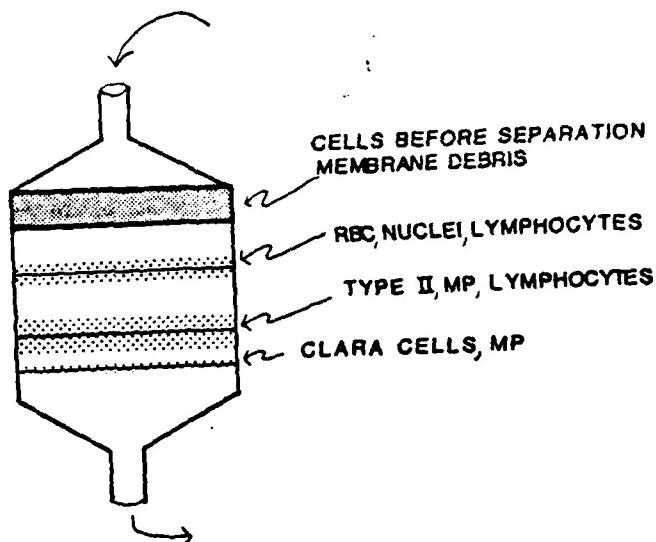
$$V = \frac{2 \omega^2 R r^2}{9m} (\rho_{cell} - \rho_{med})$$



SEPARATION BY SIZE

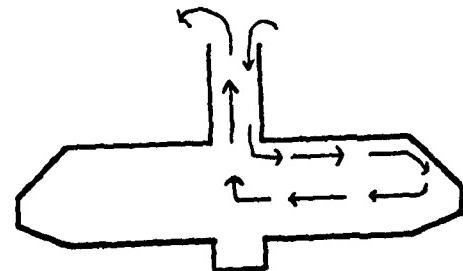
(unit gravity)

$$V = \frac{2 gr^2}{9n} (\rho_{cell} - \rho_{med})$$



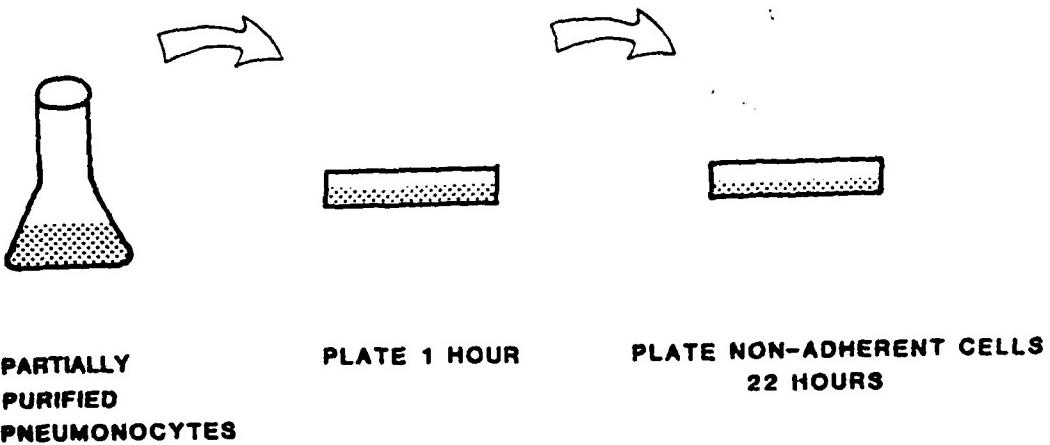
ELUTRIATION

$$V = \frac{2 \omega^2 R r^2}{9n} (\rho_{cell} - \rho_{med})$$

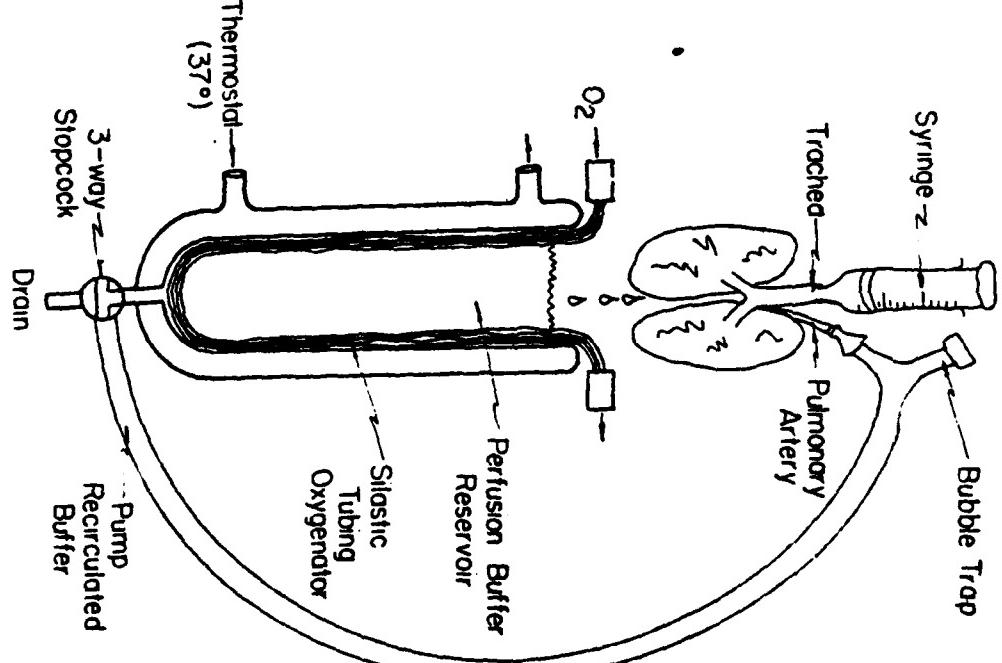


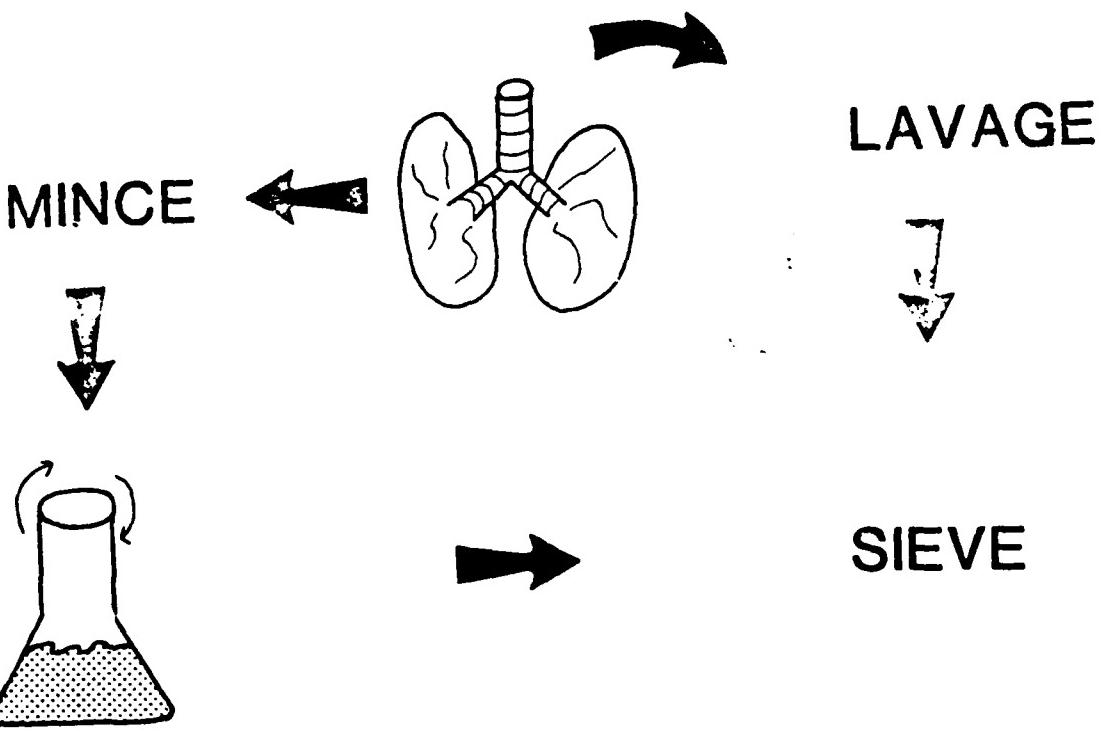
centrifugal force → ← buffer counter current

DIFFERENTIAL ADHERENCE



Perfusion Apparatus





A Buffer Reservoir
 B Pump – Flow Rate
 0.884 ml/min
 C Cell Suspension
 D 3-way Stopcock
 E Loading Chamber
 F Silastic Tubing
 G Separating Chamber
 H Funnel
 I Collecting Tube

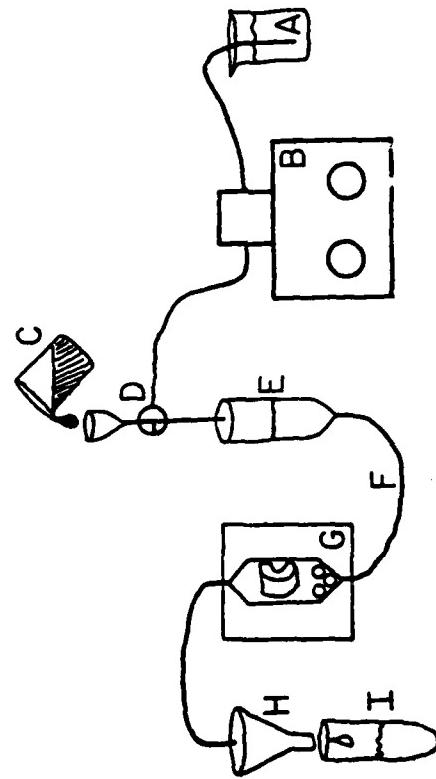
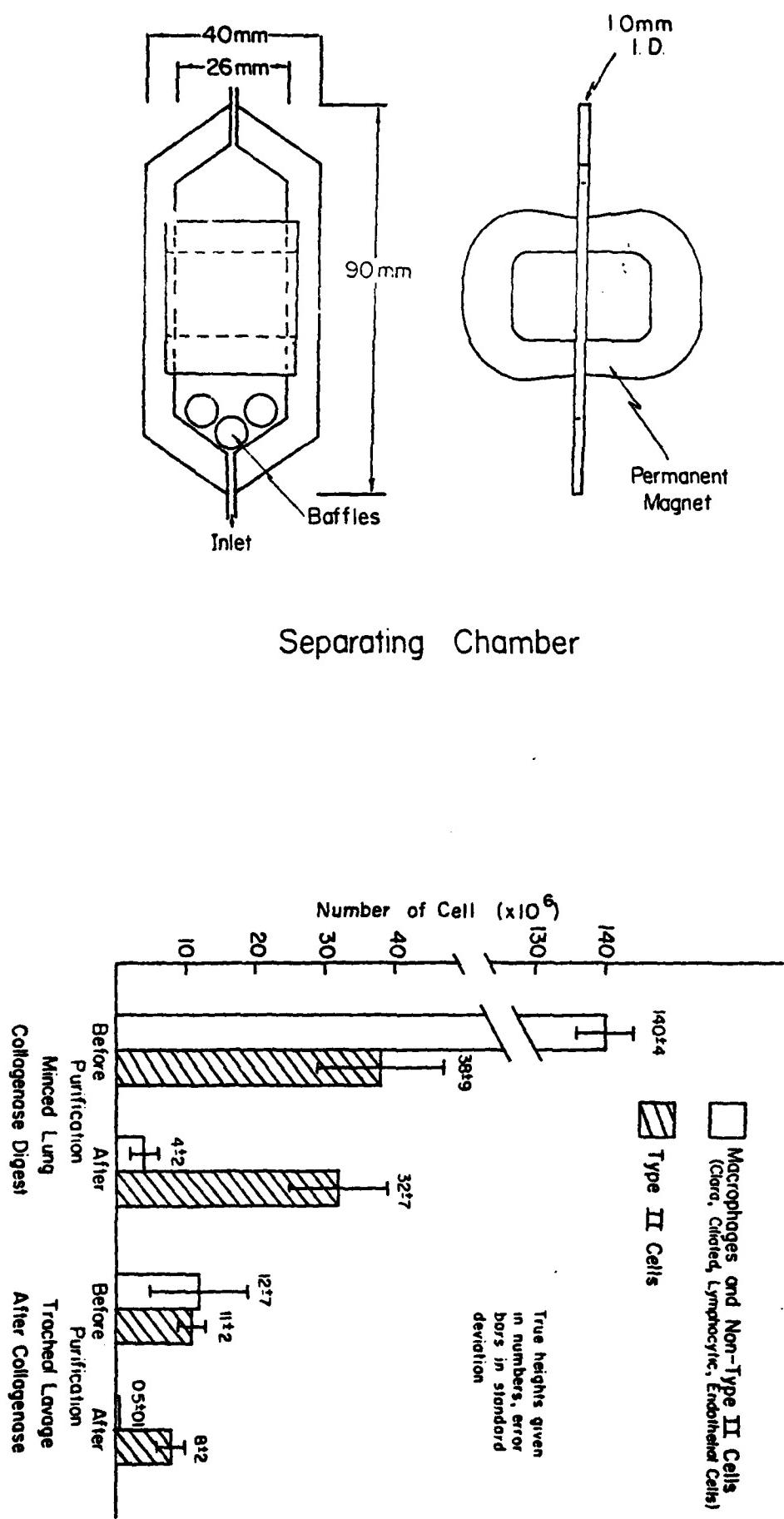
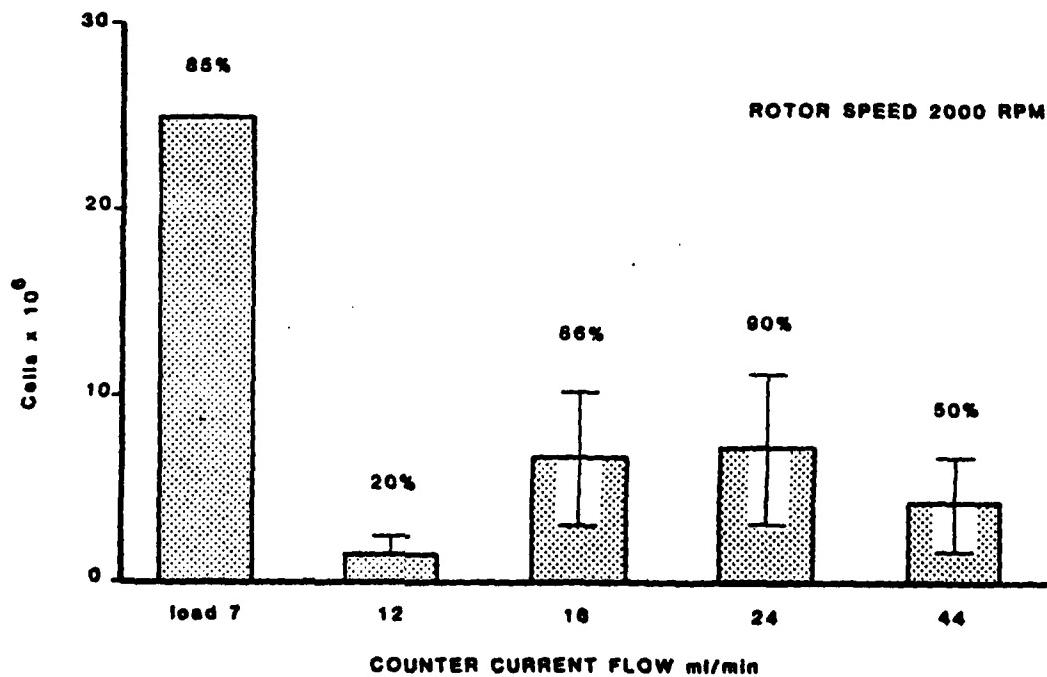


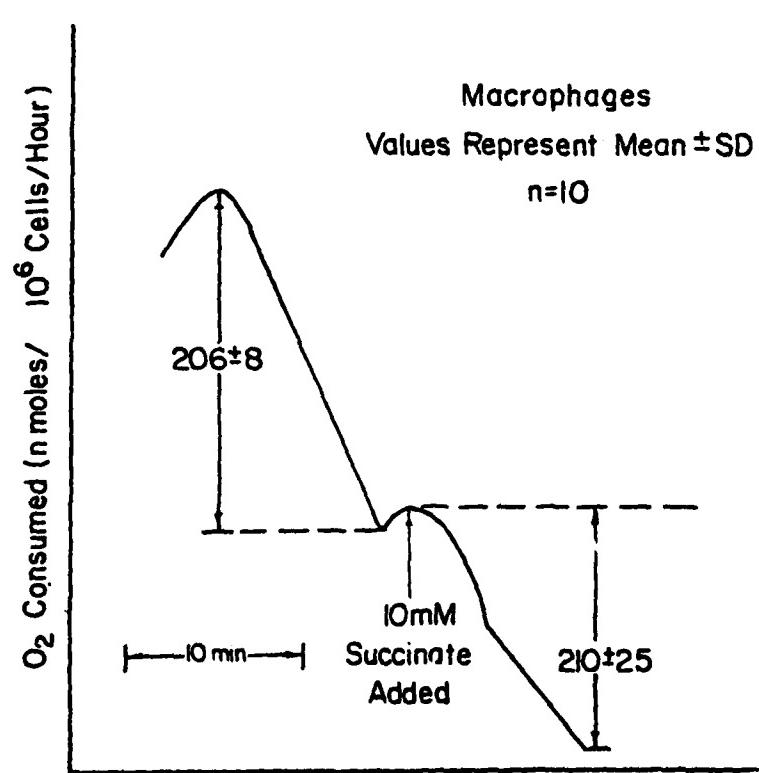
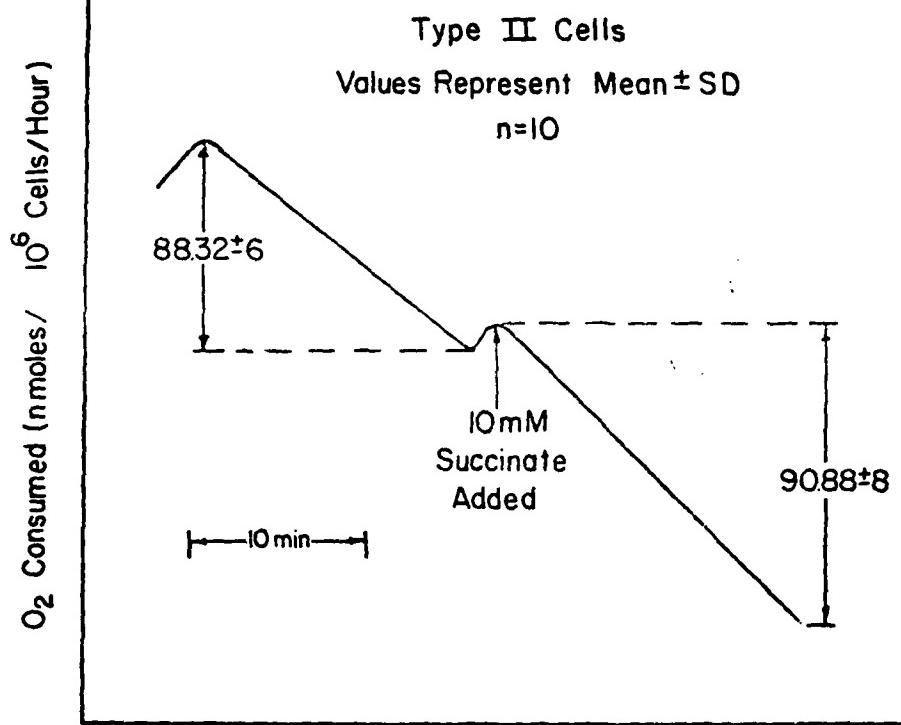
Figure 4

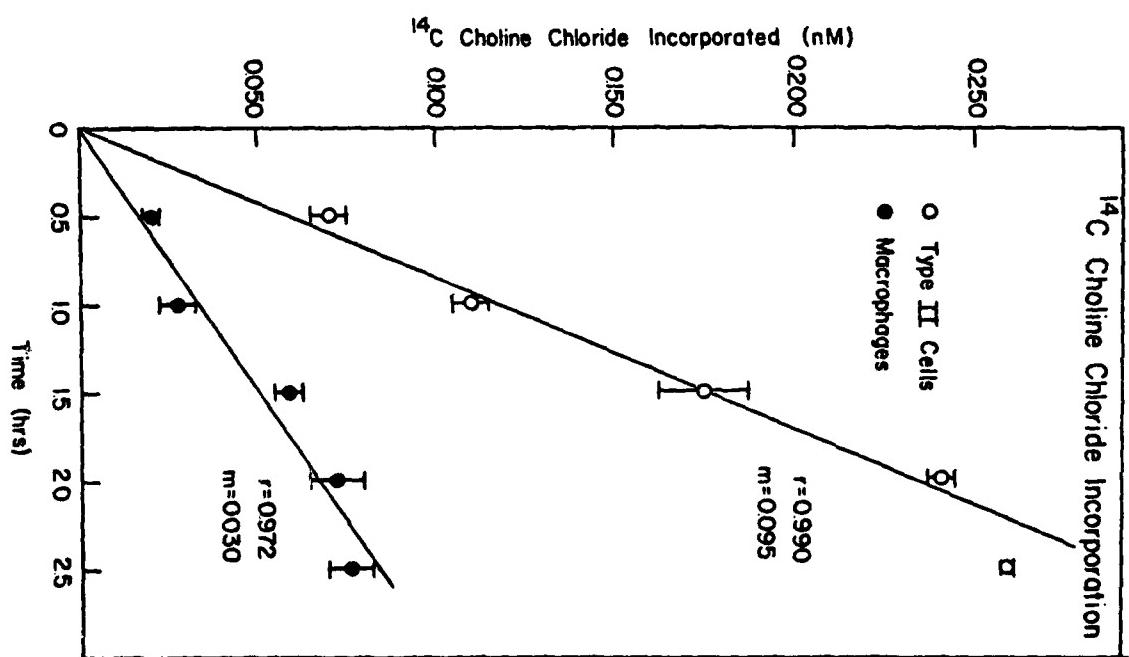
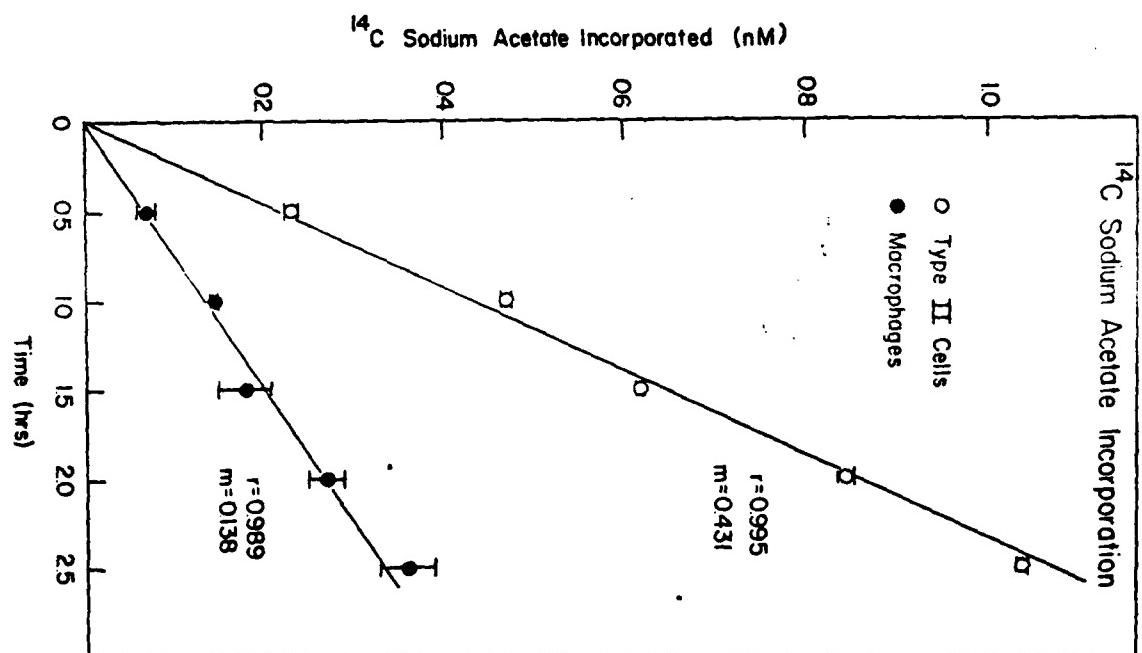




ELUTRIATION SEPARATION OF TYPE II CELLS







PRESENTATIONS

In addition to the publications and manuscripts cited in this report, several presentations were also made with support from this project. The abstracts from these presentations are included on the next three pages.

Kuttan, R., Spall, D., Sipes, I.G., Meezan, E. and Brendel, K.
(1979) Effect of paraquat treatment on rat kidney basement
membrane. *Toxicol. and Appl. Pharmacol.* 48, A177.

355. EFFECT OF PARAQUAT TREATMENT ON RAT KIDNEY BASEMENT MEM-
BRANES. R. Kuttan, R. Spall, I.G. Sipes, E. Meezan and K.
Brendel, Dept. Pharmacol., AHSC, Univ. of AZ, Tucson, AZ.

The herbicide paraquat (methyl-viologen) has been shown to produce severe lung fibrosis in both laboratory animals and human subjects. Its effect on the kidney has been much less studied. A method has been developed in this laboratory to produce "acellular kidneys" which depends on exhaustive extraction with detergents. The superstructure composed of basement membrane remains intact in these preparations. Using this method, changes in kidney basement membrane obtained 9 days after a single dose of paraquat (I.P.25 mg/Kg) were studied. Electron microscopy showed numerous "spiky" protrusions in the luminal side of tubular basement membranes of the affected kidneys, while glomerular basement membrane remained intact. Chemical analysis of the damaged basement membrane showed increased levels of hydroxylysine, 3-hydroxyproline and cysteine, possibly indicating increased basement membrane synthesis. Parallel to this finding, the synthesis of collagenous proteins of the kidney was increased which was reflected by both elevated prolyl hydroxylase levels and incorporation of proline into extracellular matrix. (Supported by a grant from ONR N00014-77-C-0506.)

Presented at the 18th Annual Meeting of the Toxicology Society,
New Orleans, 1979.

Lafranconi, W.M., Mezzan, E., Sipes, I.G. and Brendel, K. (1980)
A rapid method for preparation of lung cell fractions
enriched in alveolar type II pneumocytes. Fed. Proc.,
FASEB 39, 366.

502

PHYSIOLOGY

A RAPID METHOD FOR PREPARATION OF LUNG CELL FRACTIONS ENRICHED IN ALVEOLAR TYPE II PNEUMOCYTES. W. Mark Lafranconi*, Elias Mezzan, I. Glenn Sipes and Klaus Brendel (SPON: Thomas J. Lindell), Univ. of Arizona, Tucson, Arizona 85726.

A method was developed for the rapid isolation of type II granular pneumocyte fractions from lung of white New Zealand rabbits by the use of magnetic iron oxide to afford separation from macrophages. In this procedure a colloidal magnetite suspension is incubated with the crude cell suspension under tissue culture conditions. Alternatively isolated lung perfused with the tissue culture medium are lavaged with colloidal magnetite before trypsinization. Macrophages having phagocytosed the magnetite particles can then be pulled out of the mixture in a strong magnetic field. Sizing of the macrophage depleted pneumocyte suspension yields an enriched type II cell preparation. Viability of the cell suspension was 90-95% as determined by trypan blue exclusion. Identification of the granular pneumocytes (type II) was accomplished by modified Papanicolaou stain, fluorescence microscopy and electron microscopy. Cellular respiration was determined with the Clark type oxygen electrode. Yield of granular type II pneumocytes in this procedure as well as their relative purity were comparable to other methods described in the literature. For pharmacological or toxicological investigations, this method might offer advantages over others since it does not require the use of costly or uncommon equipment. (Supported in part by the Office of Naval Research N00014-77-C-0506 and a training grant by N.I.H. 1-I-32-ESO 7091-01).

Presented at the 64th Annual Meeting of the Federation of American Societies for Experimental Biology, Anaheim, California, April 13-18, 1980.

Kuttan, R., Lafranconi, M., Spall, D., Sipes, I.G. Meezan, E.
and K. Brendel (1979) Selective extraction of basement
membrane from lung extracellular matrix. Fed. Proc. 38,
1338.

5873)

PHARMACOLOGY

SELECTIVE EXTRACTION OF BASEMENT MEMBRANE FROM LUNG EXTRACELLULAR MATRIX. R. Kuttan*, M. LaFranconi*, D. Spall*, I.G. Sipes, E. Meezan and K. Brendel (Spon: D.G. Johnson), Dept. of Pharmacology, Arizona Health Sciences Center, Tucson, AZ 85724.

For the preparation of lung extracellular matrix the tissue was scraped with a spatula to remove large vascular and bronchial material. Homogenization and a sieving procedure afforded additional purification. An extraction procedure involving several detergents and the action of DNA'se removed all cellular material from the lung parenchymatous pieces and left the extracellular matrix, thought to be composed of basement membrane proteins as well as soluble and insoluble collagens. To extract from this mixture the basement membrane proteins, it was pretreated with 8 M urea which extracted "salt and acid soluble" collagens. The residue was then extracted with urea-mercaptoethanol which solubilized basement membranes and left a residue of insoluble collagens of various types and origins. In addition, lung basement membrane collagen was prepared from the mercaptoethanol-urea extract by pepsin digestion. Electron microscopy, gel electrophoresis and amino acid as well as carbohydrate analyses were used to characterize the various fractions. There is a remarkable similarity in the analysis of basement membrane proteins from various species and between younger and older animals. The basement membrane collagen also showed similarities with glomerular basement membrane. Supported by USPHS Grant No. HD-10781 and ONR Grant No. N00014-77-C-0506.

Presented at the 63rd Annual Meeting of the Federation of American Societies for Experimental Biology, Dallas, Texas, April 1-10, 1979.

